IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Svend Birkelund, Gunna Christiansen, Katrine Knudsen, Per Mygind og Anne-Sofie Hebsgaard Pedersen
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For	:	Surface exposed proteins from Chlamydia pneumoniae
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Art unit	:	1645

Second Declaration of Svend Birkelund

1. I, Svend Birkelund, Sindalsvej 17, DK-8240 Risskov, Denmark, in my capacity as professor at The Department of Medical Microbiology and Immunology, DK-8000 Aarhus C, Denmark, do state and declare as follows:

2. I am one of the named inventors of the above-captioned patent application. I believe that I am a person skilled in the art to which the above-captioned application pertains.

3. I have read the Office Action dated 11 March 2005. According to the Office Action the applicant has not established extrinsic evidence before the Examiner that Melgosa's 98 kDa band was a mixture of proteins.

4. To further emphasize that the Melgosa 98 kDa band in fact contained a mixture of proteins, I have, by following the instructions described by Melgosa et al. for the separation procedure, analysed the polymorphic outer membrane proteins in the 98 kDa band of *Chlamydia pneumoniae* AR39.

4.1 Problem for analysis
The present inventors have previously characterized the 98 kDa protein complex from the outer membrane complex of *Chlamydia pneumoniae* VR1310 and cloned the genes encoding the proteins (patent number PA200100581 23.06.1997, PCT 19.06.1998). The results are described in the papers: Knudsen et al. 1999, Vandahl et al. 2001, Vandahl et al. 2002.

Melgosa et al. (1993) characterized a 98 kDa band in the outer membrane complex of *Chlamydia pneumoniae* AR39 and showed that antibodies to the protein band reacted in a species specific manner.

The genomic sequences of both *C. pneumoniae* VR1310 (Kalman et al. 1999) and AR39 (Read et al. 2000) have been determined and are almost identical with only 269 mutations in 1,230,000 bases.

Because the present inventors have shown that a band in *Chlamydia pneumoniae* VR1310 contains multiple proteins, the present inventors compared the amino acid sequences of the Pmp proteins translated from the genomes of *C. pneumoniae* VR1310 and AR39 to determine whether other amino acid sequence variations could be found.

4.2 Results

The results of the comparison of the amino acid sequence of the Pmp proteins from *C. pneumoniae* VR1310 and AR39 are shown in Table 1. The present inventors identified a variation in Pmp8 where one amino acid change was found. Specifically, at position 175 a tyrosine (T) in VR1310 is changed to an alanine (A) in AR39. All other AR39 Pmp proteins were 100% identical to their VR1310 counterparts.

To determine whether the Pmp proteins were expressed and whether they migrated as bands of 98 kDa the present inventors cultivated *C. pneumoniae* AR39 in HeLa cell culture and purified the elementary bodies (EB) (Knudsen et al. 1999).

The outer membrane complex (OMC) was purified by sarcosyl extraction of the purified EB (Melgosa et al. 1993, Knudsen et al. 1999) and the proteins were separated by SDS-PAGE and silver stained. The result is shown in Figure 1A. As seen in the publication of Melgosa et al. (1993) four bands (98, 60 doublet, 39.5 (MOMP) and 15.5 kDa were found in the OMC of *C. pneumoniae* AR39. The present inventors enlarged the upper band of 98 kDa (Figure 1B), this shows that the band is composed of several bans of nearly identical size. To identify whether Pmp proteins migrated to that position immunoblotting was performed. The results are shown in figure 2. *C. pneumoniae* EB proteins were separated by SDS-PAGE and the proteins were transferred to a nitrocellulose membrane. The membrane was cut into strips. Each strip was then reacted with an antibody raised against the N-terminal part of *C. pneumoniae* Pmp protein 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 18, 19, 20 and 21. Antibodies to Pmp3 and 17 were omitted because the genes were truncated.

By computer search the theoretical molecular size of the Pmp proteins was determined (Table 1). Pmp1, 2, 7, 8, 9, 10, 11, 13, 14, 15, 16, 18 and 19 all had a size that would migrate to 98 kDa. Pmp6 is cleaved and one of the fragments has the size of 94 kDa (Vandahl et al. 2001) which also could migrate as 98 kDa. Pmp12 has the size of 56 kDa.

Vandahl et al. (2002) made antibodies to recombinant Pmp proteins and characterized them by 2D-gel immunoblotting and showed that the antibodies reacted to the proteins against which they were raised. Some cross reaction was

seen for the antibodies, when used with high antigen concentration in the 2D-gel blotting. In these experiments the present inventors reduced the cross reaction by using small amount of antigen and diluting the secondary antibody to 1:30,000 instead of the 1:2000 used by Vandahl et al. (2002). To increase the specificity the antibody reaction was performed in 500 mM NaCl, as it was done in Vandahl et al. (2002) experiments.

From figure 2 it is seen that antibodies to Pmp1, 2, 7, 8, 9, 10 and 11 are found to react with 98 kDa bands of slightly different size. The antibody to Pmp6 is seen to react with a band slightly smaller as predicted from the size of the determined fragment and the antibody to Pmp12 is shown to react with a band of 56 kDa in agreement with its predicted size.

The antibodies produced clearly different patterns, showing the antibodies could differentiate among the proteins in the 98 kDa band.

4.3 Data

Pmp	Omp	Mw (Da) VR1310	No. aa	Mw (Da) AR39	acc. nr. AR39	%homology
Pmp1	Omp6	100,370	923	100.370	NP_445309.1	100%
Pmp2	Omp7	89,600	842	89,600	NP_445300.1	100%
Pmp3*	Omp8*	truncated				
Pmp4*	Omp9*	truncated				
Pmp5*	Omp15*	truncated				
Pmp6	- - -	132,126	1408	132.126	NP_444858.1	100%
Pmp7	Omp12	100,106	937	100,106	NP_444857.1	100%
Pmp8	Omp11	97,670	931	97,640	NP_444856.1	99.8%
Pmp9	Omp10	98,333	928	98,333	NP_444855.1	100%
Pmp10	Omp5	97,229	928	97,229	NP_444852.1	100%
Pmp11	Omp4	101,357	929	101,357	NP_444851.1	100%
Pmp12	Omp13	56,095	515	56,095	NP_444850.1	100%
Pmp13	Omp14	105,600	974	105,600	NP_444848.1	100%
Pmp14	- - -	103,654	979	103,654	NP_444847.1	100%
Pmp15	- - -	102,194	939	1021,94	NP_444835.1	100%
Pmp16	- - -	104,196	935	104,196	NP_444834.1	100%
Pmp17	- - -	truncated				
Pmp18	- - -	103,611	893	103,611	NP_444833.1	100%
Pmp19	- - -	103,642	948	103,642	NP_444764.1	100%
Pmp20	- - -	180,632	1724	180,632	NP_444763.1	100%
Pmp21	- - -	170,865	1610	170,865	NP_445435.1	100%

Table 1

Comparison of the amino acid sequence of the *C. pneumoniae* VR1310 and AR39 Pmp proteins. number of amino acids, molecular size and accession numbers of the AR39 Pmp proteins are shown.

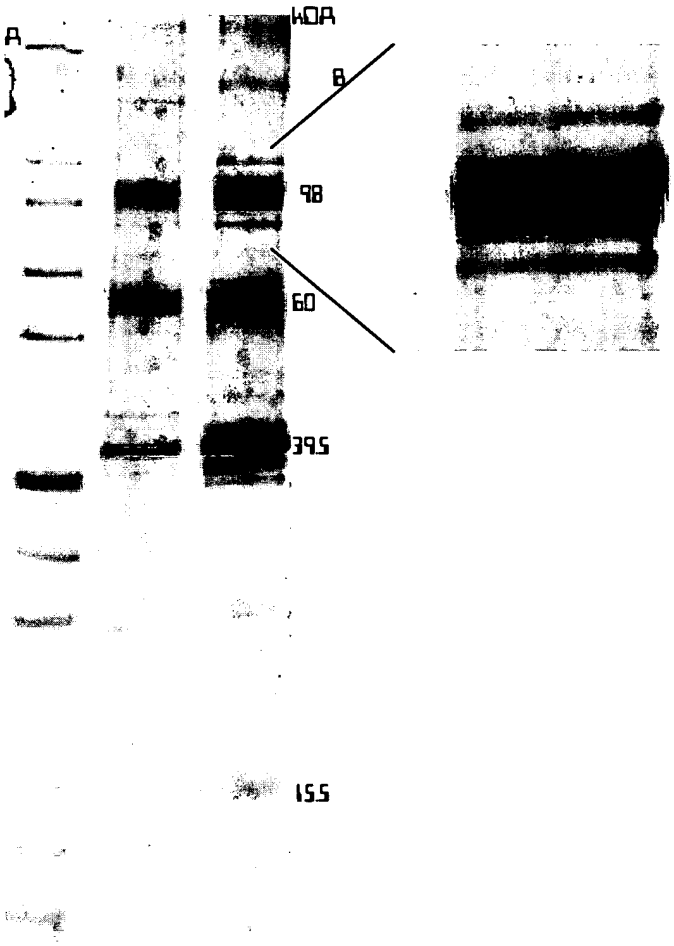
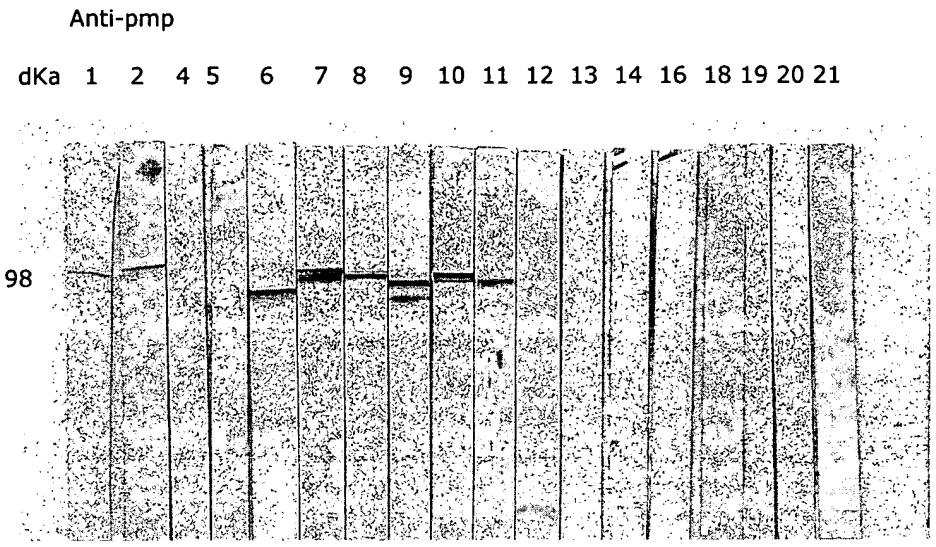


Figure 1: Silever staind 10% SDS-PAGE of purified C. pneumoniae AR39 outer membran complex (OMC). lane 1: Mark12 protein standard lane 2: COMC 0.25 microgram lane 3: COMC 0.5 microgram

Figure 2



Immunoblotting. Purified C. pneumoniae AR39 EB proteins are separated by SDS-PAGE and transfered to a nitrocellulose membrane. The membrane was cut into strips that were reacted with antibodies to Pmp 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 18, 19, 20 and 21 raised against the respective recombinant proteins.

4.4 Conclusion

By computer analysis, the present inventors compared the amino acid sequences of the Pmp proteins of *C. pneumoniae* VR1310 and AR39 and found that all were identical except for one amino acid change in Pmp8.

By silver staining of *C. pneumoniae* AR39 OMC the present inventors confirmed the findings of Melgosa et al. (1993) identifying four major bands. Enlargement of the 98 kDa band showed that it was composed of several bands of nearly identical size. By immunoblotting with antibodies raised against recombinant fragments of Pmp proteins the present inventors identified that 8 of the anti-Pmp antibodies reacted with bands of close to 98 kDa confirming that several of the Pmp proteins are present in that band in agreement with what is shown for *C. pneumoniae* VR1310 (Knudsen et al. 1999) and in agreement with the 2D mapping of the migration of Pmp proteins (Vandahl et al. 2001).

5. On the basis of the foregoing, it is clear that the single 98 kDa band reported by Melgosa et al. 1993 was actually composed of several different Pmp proteins, and hence none of these proteins was obtained free of other outer membrane proteins.

6. I further declare that all statements made herein of my own knowledge are true and further that the statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any patent issued thereon.

Dated: 9/9/2005 Signature: Svend Birkelund

Svend Birkelund

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Identification of Two Novel Genes Encoding 97- to 99-Kilodalton Outer Membrane Proteins of *Chlamydia pneumoniae*

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Two genes encoding 97- to 99-kDa *Chlamydia pneumoniae* VR1310 outer membrane proteins (Omp4 and Omp5) with mutual similarity were cloned and sequenced. The proteins were shown to be constituents of the *C. pneumoniae* outer membrane complex, and the deduced amino acid sequences were similar to those of putative outer membrane proteins encoded by the *Chlamydia psittaci* and *Chlamydia trachomatis* gene families. By use of a monospecific polyclonal antibody against purified recombinant Omp4, it was shown that without heating, the protein migrated at 65 to 75 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoelectron microscopy showed that epitopes of Omp4 were exposed on the surface of *C. pneumoniae* elementary bodies, reticulate bodies, and outer membrane complex. Proteins encoded by the *C. pneumoniae* gene family seem to be dominant antigens in experimentally infected mice.

Chlamydia pneumoniae is a widespread pathogen of the upper respiratory tract. About 10% of cases of community-acquired pneumonia have been associated with *C. pneumoniae*, but asymptomatic or mildly symptomatic infections are also common results of current infection. Other illnesses associated with this bacterium are bronchitis, pharyngitis, sinusitis, otitis media, and fever of undetermined origin (15, 37).

Saikku et al. (36) were the first to report that chronic *C. pneumoniae* infection may be associated with cardiovascular diseases. By using the microimmunofluorescence test, they showed that sera from patients with acute myocardial infarction or chronic coronary heart disease contained higher levels of antibodies to *C. pneumoniae* TWAR than did sera from control patients (36). Other studies have shown that *C. pneumoniae* may be associated with disease of the coronary or carotid arteries (16, 23, 35).

The only well-characterized surface exposed component of *C. pneumoniae* is the genus-specific lipopolysaccharide epitope (9). Several studies have been performed to identify surface exposed and immunogenic proteins. Species-specific, immunogenic proteins of 98, 53, 46, and 43 kDa have been described (8, 20, 21), and immunoelectron microscopy studies have shown that the 53-kDa protein is located on the surface (31). However, none of these proteins are good markers for *C. pneumoniae* infection, because their recognition varies among patient serum samples (24). Species-specific monoclonal antibodies (MAbs) that react with the surface of *C. pneumoniae* elementary bodies (EBs) and with the outer membrane complex (OMC) in immunoelectron microscopy have been generated, but attempts to characterize the antigenic determinant by immunoblotting have been unsuccessful (9, 34).

Sarkosyl treatment of *C. trachomatis* EBs leaves a Sarkosyl-insoluble fraction named *Chlamydia* outer membrane complex (COMC) (6) in which the major outer membrane protein (MOMP) of 38 to 42 kDa is the dominant protein (18, 38). Moreover, COMC contains the cysteine-rich outer membrane protein 2 (Omp2) doublet of 60 to 62 kDa and the cysteine-rich outer membrane protein 3 (Omp3) of 12.5 to 15.5 kDa (1, 10). In addition to MOMP, Omp2, and Omp3, the protein profiles of *C. pneumoniae* and *C. psittaci* OMC contain proteins of approximately 98 kDa which are not seen in *C. trachomatis* OMC (8, 28, 30).

In the *C. psittaci* ovine abortion strain, a 98-kDa OMP migrated at 66 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without heating (28). In later studies of the same strain, Longbottom et al. (27) identified five genes, named OMP90 gene family, encoding homologous OMPs of 90 to 98 kDa. Immunoblotting with postabortion sheep sera showed that these proteins, and especially the amino-terminal ends, were major immunogens (27). In addition, the protein family could be located to the surface of both EBs and reticulate bodies (RBs) by immunoelectron microscopy (26). This is in contrast to results obtained by Buendia et al. (5) which showed that a group of 80- to 90-kDa proteins from *C. psittaci* serotype 1 AB7 was located on the surface of RBs but not EBs.

The aim of this study was to identify the genes encoding the 97- to 99-kDa proteins present in the *C. pneumoniae* VR1310 OMC, to analyze the localization and antigenicity of the proteins, and to compare the gene sequences with those of the gene family from *C. psittaci* encoding the group of 90- to 98-kDa proteins.

MATERIALS AND METHODS

***C. pneumoniae* strain and cultivation conditions.** *C. pneumoniae* (CDC/CWL-029/VR-1310), purchased from the American Type Culture Collection (Rockville, Md.), was cultivated for 72 h in HeLa 229 cells (American Type Culture Collection) as described previously (11).

Enzymes and primers. The restriction enzymes and enzymes used for PCR and cloning were purchased from Boehringer GmbH (Mannheim, Germany). DNase I (grade II; bovine pancreas), and RNase was obtained from Worthington Biochemical Corp. (Freehold, N.J.). DNA polymerase I was obtained from Gibco BRL Life Technologies (Gaithersburg, Md.), and Benzoase was obtained from Sigma (St. Louis, Mo.). Primers used for sequencing and PCR were purchased from DNA technology (Aarhus, Denmark).

Purification of *C. pneumoniae*. EBs were purified from HeLa cells grown in 16 six-well plates (Nunc, Roskilde, Denmark). The infected HeLa cells were disrupted by sonication for 30 s. HeLa cell debris was removed by centrifugation (4 min at 200 × *g*). The supernatant was then ultracentrifuged for 1 h at 100,000 × *g* through a layer of 30% Urografin (Schering-Plough Corp., Madison, N.J.) and a layer of 50% sucrose. The pellet was dissolved in HEPES buffer (10 mM HEPES, 150 mM NaCl [pH 7.2]), sonicated briefly, and digested with 50 μg of

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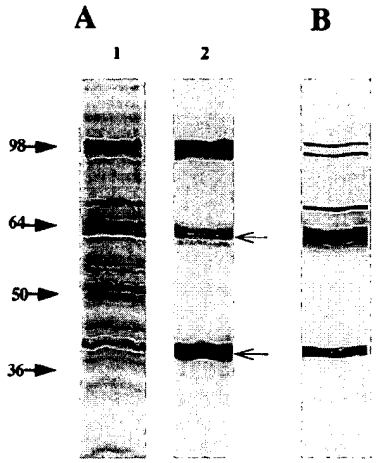

FIG. 1. (A) Silver-stained SDS–10% polyacrylamide gel of *C. pneumoniae* EB (lane 1) and OMC (lane 2). The samples were boiled in SDS sample buffer. (B) Immunoblotting of *C. pneumoniae* EBs reacted with the polyclonal rabbit antiserum to *C. pneumoniae* OMC. The arrows indicate the migration of Omp2 (60 to 62 kDa) and MOMP (39.5 kDa).

RNase per ml and 40 μg of DNase per ml. The suspension was ultracentrifuged for 1 h at 200,000 × *g* through a discontinuous gradient consisting of 34, 40, 46, and 52% Urografin (Schering-Plough). Upon centrifugation, the three layers (an EB layer, an intermediate layer, and an RB layer) were transferred to separate vials, diluted in HEPES buffer, and ultracentrifuged for 30 min at 200,000 × *g*. Finally, supernatant was discharged, and each pellet was resuspended in HEPES buffer and stored at −70°C.

Purification of the *C. pneumoniae* OMC. EBs were dissolved in phosphate-buffered saline (PBS) containing 2% sodium *N*-lauroylsarcosine (Sarkosyl) and 5 mM EDTA, sonicated briefly, and incubated for 30 min at 37°C. Insoluble material was pelleted by centrifugation. The pellet was dissolved in PBS containing 2% Sarkosyl and 5 mM $MgCl_2$, sonicated briefly, and digested with Benzoase and RNase for 30 min at 37°C. Insoluble material was pelleted, washed twice in PBS, and resuspended in PBS.

SDS-PAGE, immunoblotting, and silver staining. The concentrations of *C. pneumoniae* EB and OMC were estimated from a silver-stained SDS-PAGE gel, and about 1 μg was applied per lane. The concentration of recombinant Omp4 was measured with the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, Ill.) as specified by the manufacturer. Approximately 2 μg was applied per lane.

C. pneumoniae EB and OMC solubilized in SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 2.3% [wt/vol] SDS, 10% [wt/vol] glycerol, 5% [wt/vol] β-mercaptoethanol, 0.05% [wt/vol] bromphenol blue) were either heated to 100°C for 5 min or incubated at room temperature for 15 min, separated by SDS-PAGE (25), and electrophoretically transferred to nitrocellulose membranes. Electrotransfer and immunodetection were carried out by a protein-blotting procedure (Bio-Rad, Richmond, Calif.).

MAb 24.1.44 was generated against purified *C. pneumoniae* EBs and shown by immunoelectron microscopy to react with the *C. pneumoniae* surface (9). For immunoblotting, MAb 24.1.44 was diluted 1:3 (tissue culture supernatant) and the polyclonal antisera were diluted 1:200 in antibody buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, 0.2% gelatin [pH 7.5]). Bound polyclonal antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Bio-Rad). Bound MAb 24.1.44 was detected with alkaline phosphatase-conjugated rabbit anti-mouse IgG (Bio-Rad). The blots were stained with 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium (BCIP-NBT) color development solution (Bio-Rad).

Silver staining of the proteins separated by SDS-PAGE was performed by the method of Sambrook et al. (39). Negative Cu^{2+} staining of the polyacrylamide gel, excision of the band, and extraction of proteins were done as described previously (28).

Production of a polyclonal rabbit antiserum against *C. pneumoniae* OMC. For each immunization, 10 μg of antigen was incubated for 15 min with 30 μl of SDS sample buffer, diluted in PBS, and emulsified in Freund's incomplete adjuvant (Difco Laboratories, Detroit, Mich.). A New Zealand White rabbit was immunized intramuscularly on days 1 and 7 with SDS-treated antigen. On day 20 and 27, it was immunized intravenously with antigen in PBS. The rabbit was bled on day 69.

Cloning of *C. pneumoniae* DNA, production of libraries, and screening. Genomic DNA was extracted from *C. pneumoniae* (29), centrifuged on a CsCl gradient, and dialyzed against TE buffer (39). For the expression library, the DNA was partially digested with *Sau*3AI and cloned in pEX1, pEX2, and pEX3 vectors (40), and the expressed peptides were screened by colony blotting with the polyclonal antiserum to denatured *C. pneumoniae* OMC as previously described (3).

For the production of a genomic library, 1 μg of genomic *C. pneumoniae* DNA was completely digested with 10 U of *Bam*HI and cloned in the pBluescript vector (Stratagene, La Jolla, Calif.). Recombinant plasmids were electrotransformed into competent *E. coli* XL1-blue cells, and the colonies were blotted onto nitrocellulose (39). A 410-bp fragment (which covers bp 568 to 978 of *omp5*) was amplified by PCR on genomic *C. pneumoniae* DNA with two primers 5′-GGG AATAAATCGAGCGC-3′ and 5′-CAGCTGCCAGTATAGAAATGG-3′ and used for production of a radioactive probe ([α-^{32}P]dATP was obtained from Amersham International, Little Chalfont, United Kingdom). The genomic library was screened as described previously (39).

The plasmid DNA was extracted from the recombinant *E. coli* by the alkali lysis method (39), except that an additional step of lysozyme digestion was included. When the plasmid DNA was used for automatic sequencing, the step involving phenol extraction was omitted.

DNA sequencing and sequence analysis. Template DNA (0.5 μg) was mixed with primer and Thermo Sequenase (Amersham, Cleveland, Ohio) and thermocycled in a DNA thermal cycler as described by the manufacturer (Gene-Amp PCR system 9600; Perkin-Elmer, Warrington, United Kingdom). Sequencing was performed in a ABI PRISM Dye Terminator apparatus (Perkin Elmer). The sequences were analyzed with the programs in the Wisconsin Package, version 8.1 (Genetics Computer Group [GCG], Madison, Wis.).

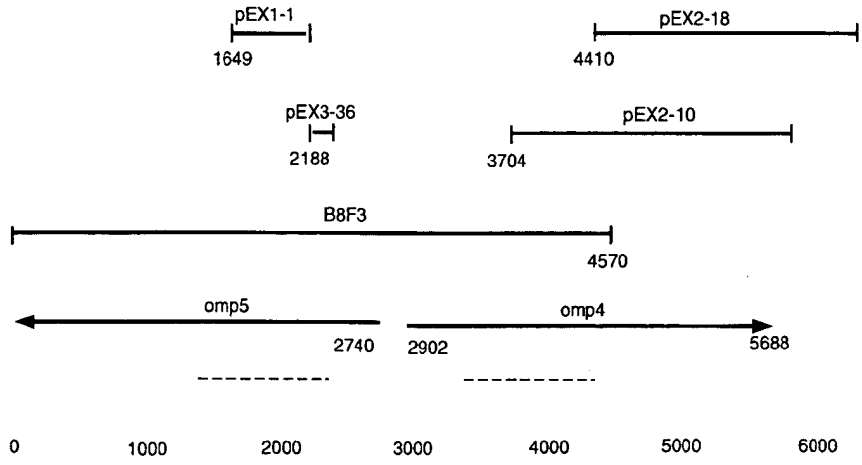

FIG. 2. Diagram of the overlapping pEX clones and pBluescript clones. The two open reading frames that were named *omp4* and *omp5* are shown (the arrows indicate the direction of transcription). Dotted lines show the regions where the nucleotides encoding the repeated amino acid motifs (GGAI) were found. Base numbers are designated at the bottom.

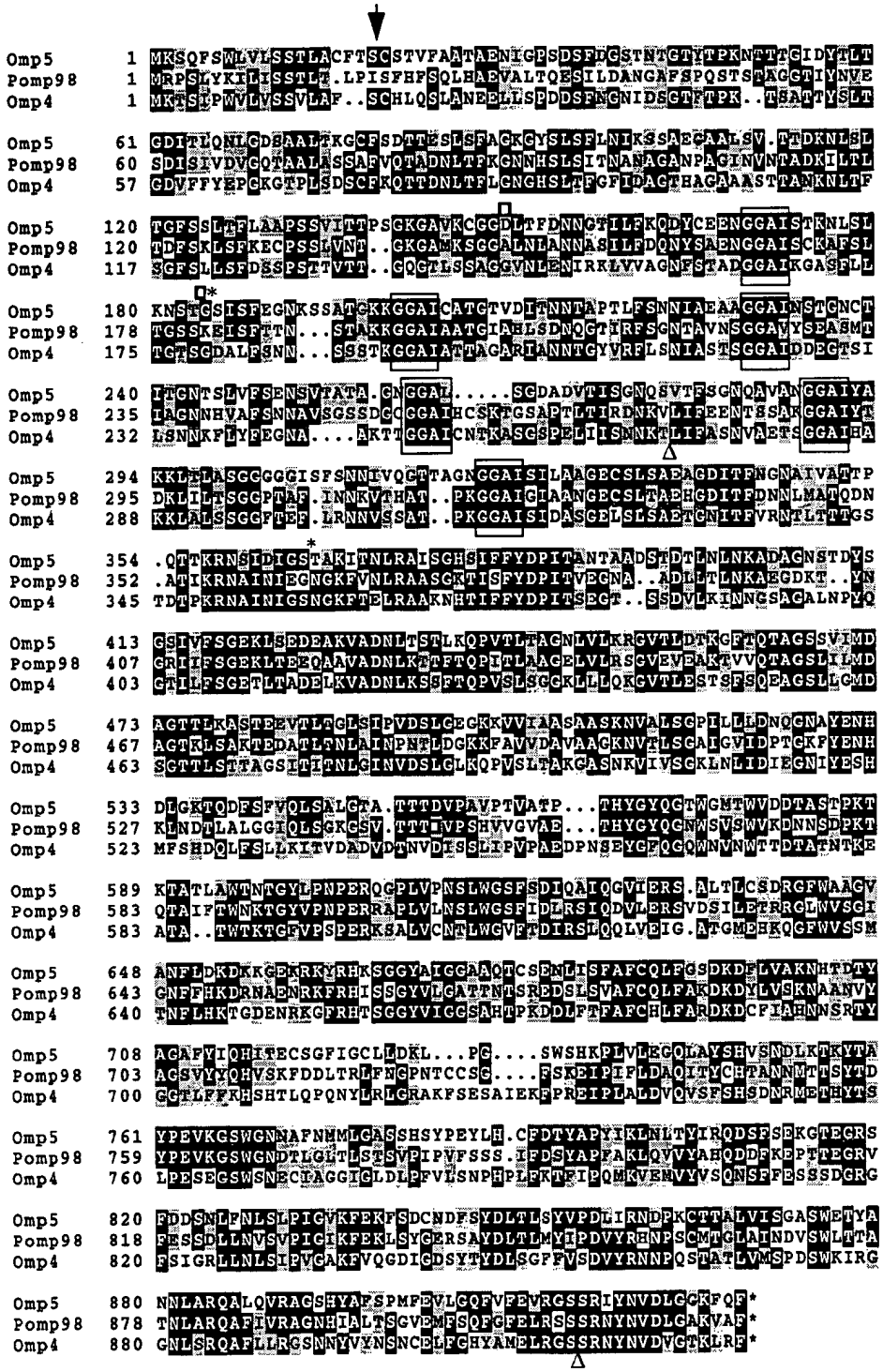

FIG. 3. Multiple alignment of *C. pneumoniae* Omp4 and Omp5 and *C. psittaci* Pomp98 (27). Amino acid sequence alignment was done with the PILEUP program (GCG 8.1 package). Dots represent gaps, the black areas indicate amino acid identity, and the grey areas indicate amino acid similarity. The repeated amino acid motifs are boxed. The arrow shows the putative signal peptidase II cleavage sites in *C. pneumoniae* Omp4 and Omp5. The beginnings and ends of peptides expressed from the pEX clones are indicated by boxes (pEX3-36), asterisks (pEX1-1), and triangles (pEX2-10), respectively.

High-fidelity PCR. By use of the high-fidelity PCR kit (Boehringer GmbH), a fragment containing the 3′ end of the *omp5* gene was amplified on genomic *C. pneumoniae* DNA with the primers 5′-GGCAGTCACTACGCCTTCTCTCCT ATGTTTGAAGTGC-3′ (located at the 5′ end of the B8F3 fragment) and 5′-GCCTCCGAAGACAATATAAGGTACCGTCATAACAGCGG-3′ (located in an expression clone [pEX3-29] that encodes a peptide with similarity to Omp4 and Omp5). A fragment comprising the *omp4* and *omp5* genes was amplified on genomic DNA with the primers 5′-GCACTTCAAACATAGGAG AGAAGGCGTAGTGACTGC-3′ and 5′-CCCTCAGTAAAAATGCCTGTCT TGAAAGATTGCCACCG-3′.

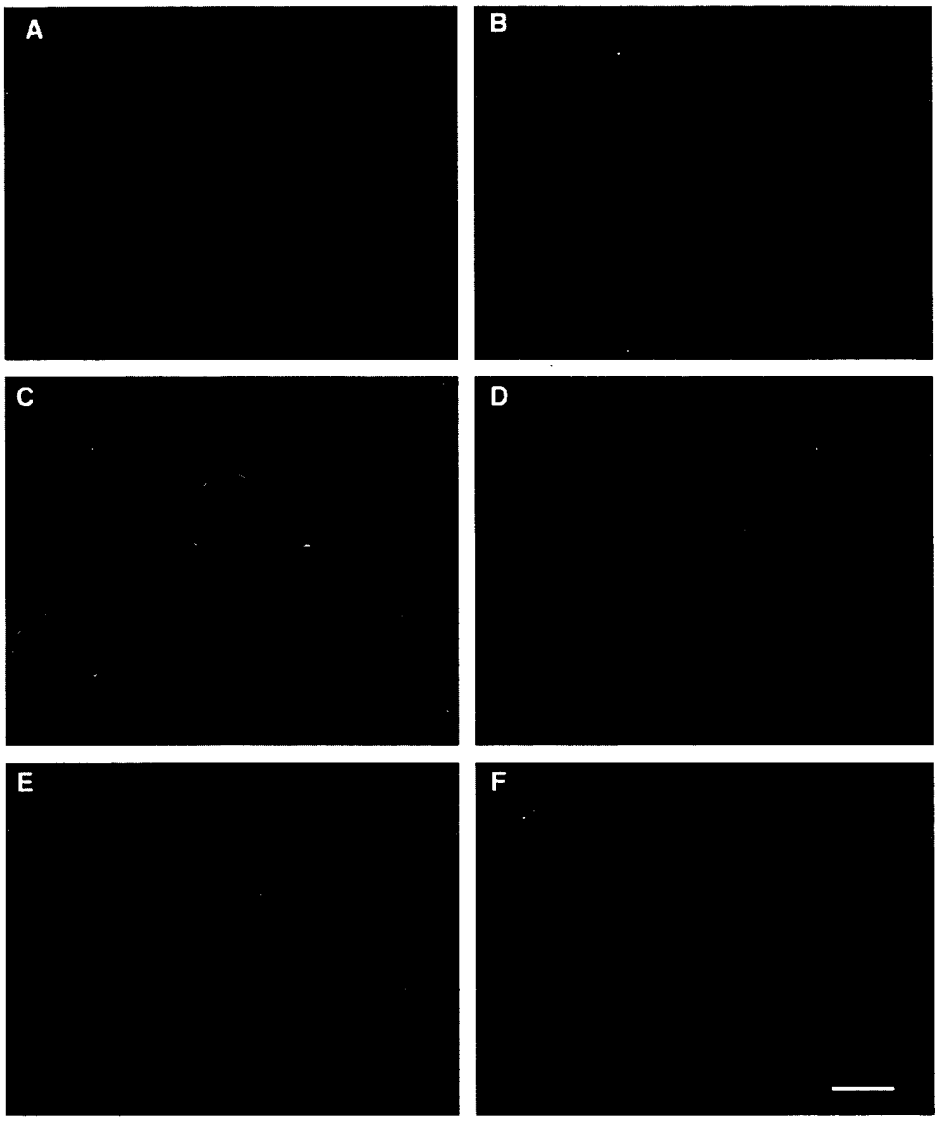

FIG. 4. IMF of *C. pneumoniae* inclusions. *C. pneumoniae*-infected HeLa cells were grown for 72 h, fixed with 3.7% formaldehyde (A, B, and D through F) or methanol (C), and reacted with antibodies. (A) PAb-Omp4$_{271-914}$; (B) PAb-Omp5$_{149-184}$; (C and D) MAb 18.1 (against DnaK); (E) normal mouse serum; (F) MAb 24.1.44 (9). Bar, 10 μm.

Purification of inclusion bodies and production of mouse polyclonal antibodies. Fusion proteins from the pEX clones were expressed in *E. coli*, and the recombinant *C. pneumoniae* proteins were extracted as described previously (17). For immunization of BALB/c mice, the proteins were dissolved in PBS and emulsified in Freund's incomplete adjuvant (Difco). Immunization was performed intramuscularly on days 1, 15, 22, and 35 with 50 μg of protein each time. The mice were bled on day 85.

Production of a hyperimmune antiserum against recombinant Omp4. The *omp4* gene was amplified by PCR with the primers 5'-GACGACGACAAGAT GAAGACTTCGATTCCTTGGGTTTTAGTTTCC-3' and 5'-GAGGAGAAG CCCGGTTAGAATCGGAGTTTGGTACCAACATCTACATTG-3', which contained LIC sites, and the PCR products were cloned into the pET-30 LIC vector (Novagen, Madison, Wis.) as described by the manufacturer. The histidine-tagged fusion protein was expressed and purified, and a New Zealand White rabbit was immunized six times intramuscularly (with the antigen dissolved in PBS and emulsified in Freund's incomplete adjuvant) and three times intravenously (with the antigen dissolved in PBS) with 8 μg of protein each time. The rabbit was bled on day 60.

IMF. HeLa cell monolayers were infected with 0.5 inclusion-forming unit of *C. pneumoniae* per HeLa cell and cultivated for 72 h. Indirect immunofluorescence microscopy (IMF) was performed as described previously (11), except that when the cells were incubated with the mouse polyclonal antibodies (PAbs), 20% fetal calf serum (Gibco) in PBS was used as the antibody buffer. Mouse PAbs were used at 1:100, and MAbs (tissue culture supernatant) were used at 1:10.

Immunoelectron microscopy. A 10-μl drop of purified *C. pneumoniae* EB, RB, or OMC suspension was placed for 1 min on top of a 400-mesh nickel grid covered with freshly glow-discharged carbon film. After being washed in 3 drops of PBS, the grid was incubated for 5 min at room temperature with PBS containing 0.5% ovalbumin, transferred to a drop containing the MAb, and incubated for 30 min at 37°C. MAb 24.1.44 (tissue culture supernatant) was diluted 1:5 in PBS–0.5% ovalbumin. As the secondary antibody, 10-nm-colloidal-gold-labeled rabbit anti-mouse Ig (British Biocell, Cardiff, United Kingdom) diluted 1:20 in PBS with 1% cold fish gelatin was used. The grids were stained with 2 drops of 0.3% phosphotungstic acid (pH 7) and blotted dry. Electron microscopy was done with a JEOL 1010 transmission electron microscope at 40 kW.

Experimental infection of C57 black mice. Four C57 black mice were inoculated intranasally with 10^7 inclusion-forming units of *C. pneumoniae* under a light ether anaesthesia (32). After 14 days of infection, the serum samples were obtained and used for immunoblotting (1:100).

Nucleotide sequence accession numbers. The nucleotide and amino acid sequences of *C. pneumoniae omp4* and *omp5* have been submitted to the GenEMBL database and assigned accession no. AJ001311.

RESULTS

Protein profile and antigenicity of the *C. pneumoniae* OMC. In addition to the proteins present in the *C. trachomatis* OMC,

proteins of 98 kDa have been found in the *C. pneumoniae* OMC (8, 30). To obtain antibodies reacting with these proteins, purified *C. pneumoniae* OMC was denatured with SDS and used for immunization of a rabbit. Comparison of the protein profiles of *C. pneumoniae* EBs and OMC is shown in Fig. 1A. As expected, bands corresponding in size to MOMP (39.5 kDa) and Omp2 (60 to 62 kDa) were seen in *C. pneumoniae* OMC (Omp3 was not detected because of its low molecular mass). In addition, a band of 97 to 99 kDa was detected (lane 2). By immunoblotting of EBs, it was shown that the polyclonal antiserum generated against *C. pneumoniae* OMC recognized bands corresponding in size to MOMP, Omp2, and the 97- to 99-kDa proteins (Fig. 1B). A band of 75 kDa, which probably represented DnaK (3), was also recognized.

Detection of recombinant clones. Since the polyclonal antiserum to *C. pneumoniae* OMC recognized the 97- to 99-kDa proteins, it was used to screen an expression library. A total of 49 immunoreactive clones were obtained and sequenced from the 5′ end of the cloned insert. By using the FASTA program from the GCG program package, 10 clones were shown to contain inserts of the well-known *C. pneumoniae omp* genes (*momp*, *omp2*, and *omp3*) and 7 clones were shown to represent the *dnaK* gene. The generation of DnaK antibodies is probably due to the peptide binding ability of this protein (4), whereby it was copurified in the outer membrane preparation. The other 32 clones had an insert without homology to these genes. These inserts were further sequenced until a stop codon was found in frame or the entire sequence of the insert was obtained.

Identification of two novel *C. pneumoniae* genes. To obtain the complete open reading frames encoding the proteins represented by the expression clones, a genomic *C. pneumoniae* *Bam*HI library of 1,000 clones was produced and screened by colony hybridization with a radioactive probe representing a 410-bp fragment of the pEX1-1 clone (Fig. 2). A *Bam*HI fragment of 4.5 kbp (B8F3) was obtained. Sequencing of this fragment showed that it overlapped with three additional pEX clones (pEX3-36, pEX2-10, and pEX2-18). The complete sequence of the pEX2-18 clone and the B8F3 clone comprised 6.5 kbp that contained two open reading frames (Fig. 2). These two genes were named *omp4* and *omp5*, since they most probably encoded OMC-associated proteins of *C. pneumoniae*. A potential stem-loop structure was detected 7 bp downstream of *omp4*. Due to the presence of a *Bam*HI site in the *omp5* sequence, the 3′ end of this gene was not contained within the B8F3 fragment (Fig. 2). By performing PCR with primers obtained from the sequences of the expression clones, the 3′ end of the *omp5* gene was determined. Computer analyses did not predict any stem-loop or termination structures downstream of *omp5*.

Use of PCR on genomic *C. pneumoniae* DNA with primers that annealed to the 3′ ends of *omp4* and *omp5* confirmed that the two genes are adjacent on the *C. pneumoniae* genome (results not shown).

Sequence analysis of the two OMPs Omp4 and Omp5. The two identified open reading frames, each comprising 2,784 bp, encoded two proteins, named Omp4 and Omp5, with calculated molecular masses of 98.9 and 97.2 kDa, respectively. As illustrated (Fig. 2), *omp4* and *omp5* were transcribed in opposite directions. Potential promoter elements were identified in the intergenic region between *omp4* and *omp5* (19). Potential −10 elements were identified for both genes, but a potential −35 element was identified only for *omp5*. In addition, putative ribosomal binding sites were present in both genes. The amino-terminal parts of Omp4 and Omp5 were relatively hydrophobic, and signal peptidase II cleavage sites were predicted in both proteins, which indicated lipid modification (Fig. 3).

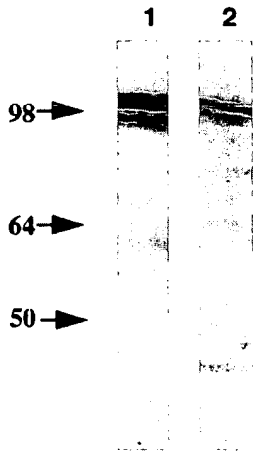

FIG. 5. Immunological identification of Omp4 by immunoblotting. Purified *C. pneumoniae* OMC was reacted with PAb_{Omp4} (lane 1), and recombinant Omp4 was reacted with MAb 24.1.44 (lane 2). The proteins were boiled in SDS sample buffer and separated by SDS-PAGE.

The sequences of *omp4* and *omp5* and the derived amino acid sequences were compared. The percentage of identical nucleotides between the two genes was 53%, and the amino acid similarity was 61%. In addition, the amino acid motif glycine-glycine-alanine-isoleucine (GGAI) was repeated six times in Omp4. Omp5 contained these repeats as well, except that in one of the repeats the isoleucine was replaced by a leucine (Fig. 3). Both Omp4 and Omp5 contained cysteine (8 and 16 residues, respectively).

Further alignment showed that two additional pEX clones encoded peptides that were similar but not identical to the Omp4 and Omp5 sequences. PCR performed on genomic *C. pneumoniae* DNA with primers located in each of the two additional pEX clones revealed that they did not represent two different regions of the same gene (results not shown). Thus, a family of at least four genes encoding homologous OMPs exists in *C. pneumoniae*.

Database search in the GenEMBL database showed that Omp4 and Omp5 were similar to the OMP90 (formerly POMP) family of five 90- to 98-kDa OMPs from *C. psittaci* (27). A DNA alignment between *omp4* and *omp5* from *C. pneumoniae* and the *pomp* genes from *C. psittaci* showed that there were 49 to 54% identical nucleotides. Moreover, the similarity between one of the *C. psittaci* proteins (Pomp98) and *C. pneumoniae* Omp5 was 63%, which is even higher than the similarity between Omp4 and Omp5. The repeated amino acid motif GGAI was conserved in Pomp98, except that in one of the repeats an isoleucine was replaced by a valine (Fig. 3).

A similar gene family encoding nine putative membrane proteins (PmpA to PmpI) of 70 to 200 kDa has also been identified in *C. trachomatis* (41). Although the repeated amino acid motif (GGAI) was found in these proteins, the overall similarity between the *C. pneumoniae* and the *C. trachomatis* membrane proteins was low.

Surface localization of Omp4 and Omp5. To determine the localization of Omp4 and Omp5 in *C. pneumoniae*, polyclonal mouse antisera to purified recombinant proteins from the pEX clones representing fragments of Omp4 and Omp5 were generated. The reaction of the mouse PAbs was investigated by IMF of infected HeLa cells fixed with formaldehyde 72 h after

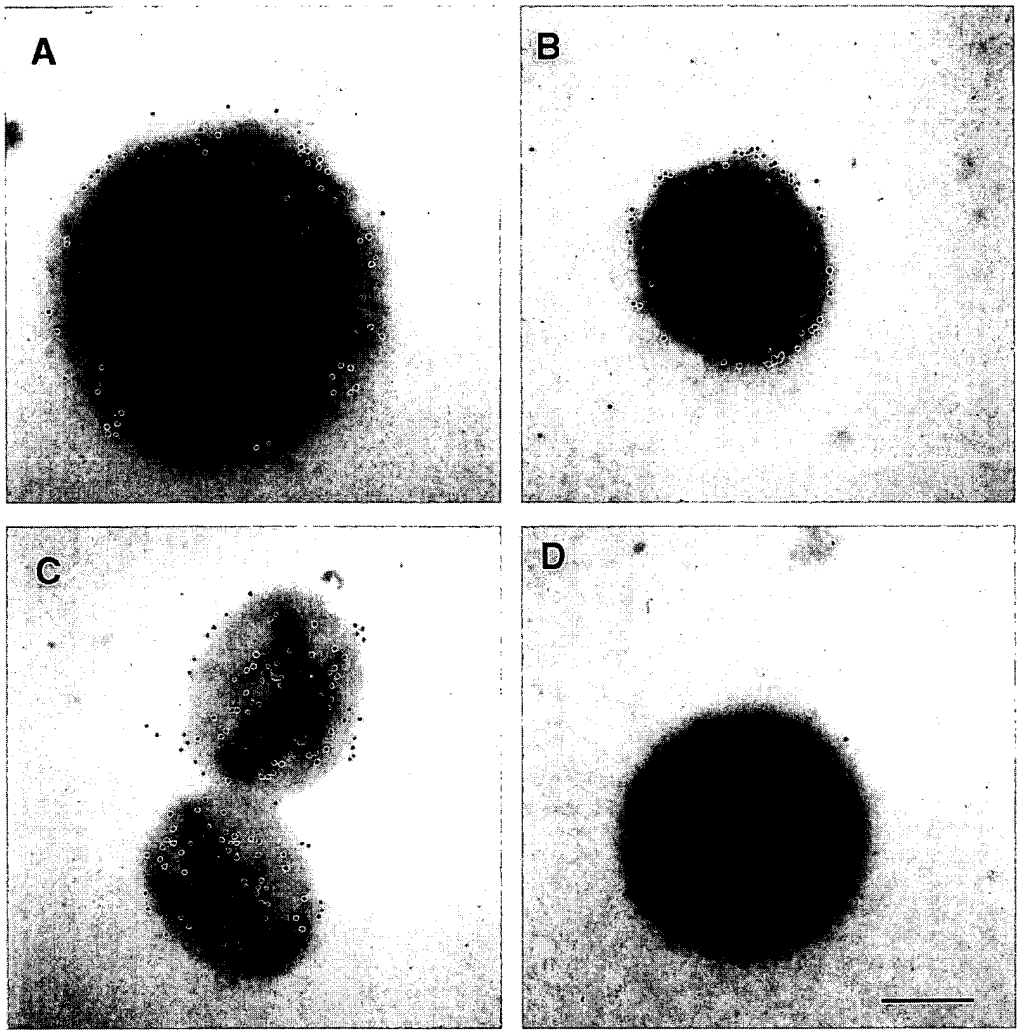

FIG. 6. Immunoelectron microscopy of *C. pneumoniae* RB (A), EB (B), and OMC (C) incubated with MAb 24.1.44 and of *C. pneumoniae* EB reacted with a MAb against *C. trachomatis* MOMP (negative control) (D). Bar, 0.2 μm.

infection. Antibodies to the peptides expressed by pEX2-10 (which covers amino acids 271 to 914 of Omp4 [Fig. 3]) and pEX3-36 (which covers amino acids 149 to 184 of Omp5 [Fig. 3]) reacted strongly in IMF with the surface of *C. pneumoniae*, as shown by the presence of fluorescent chlamydial inclusions (Fig. 4A and B). It is generally assumed that fixation of the cells with formaldehyde makes the chlamydiae impermeable to antibodies due to cross-linking of the OMPs. To verify this, MAb 18.1, which reacts with *C. trachomatis* and *C. pneumoniae* DnaK in immunoblotting (4), was also used in IMF (Fig. 4C and D). The epitope of MAb 18.1 was shown to be formaldehyde insensitive (results not shown). It reacted strongly with *C. pneumoniae* when the cells were methanol fixed and thus permeabilized (Fig. 4C) but not when the cells were fixed with formaldehyde (Fig. 4D). No reaction was seen when the infected cells were incubated with normal mouse serum (Fig. 4E). Thus, IMF showed that epitopes of Omp4 and Omp5 were exposed on the surface of formaldehyde-fixed *C. pneumoniae*.

Migration pattern of Omp4 in SDS-PAGE. When the mouse PAbs that reacted in IMF (Fig. 4A and B) were used in immunoblotting of denatured *C. pneumoniae* EB proteins, no reaction was seen, indicating that these antibodies recognized nonlinear epitopes (results not shown). We therefore amplified, cloned, and expressed the entire *omp4* gene. The recombinant Omp4 (rOmp4) was purified under denaturing conditions and used for immunization of a rabbit to obtain an antibody reacting with linear epitopes. When the hyperimmune serum against rOmp4 (PAb_{Omp4}) was used in immunoblotting of *C. pneumoniae* OMC, a strong reaction was seen with a 99-kDa band and a weak reaction was seen with a 97-kDa band (Fig. 5, lane 1). In addition, when a panel of MAbs specific for the *C. pneumoniae* surface (9) were tested for reaction in immunoblotting with rOmp4, MAb 24.1.44 reacted (lane 2). In IMF of *C. pneumoniae* inclusions, MAb 24.1.44 recognized a surface-localized epitope (Fig. 4F) that was shown by transmission immunoelectron microscopy of native, unfixed samples to be present both on the surface of RBs, EBs, and purified OMC (Fig. 6). This indicated that Omp4 had surface-exposed epitopes.

To determine the migration of Omp4 in boiled and unboiled samples, MAb 24.1.44 was reacted in immunoblots with *C. pneumoniae* OMC proteins. In the sample that was boiled prior to SDS-PAGE, a reaction with a band of 99 kDa corresponding

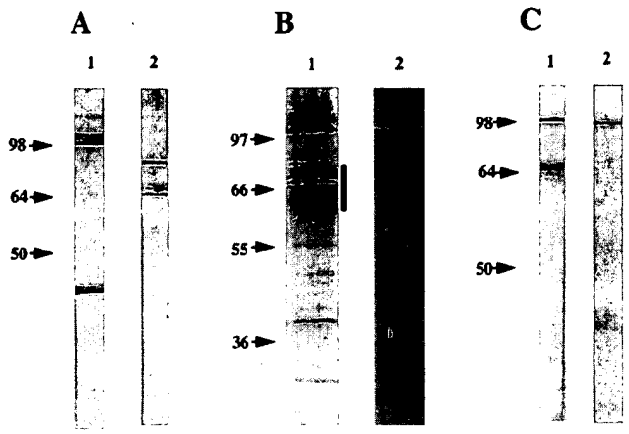

FIG. 7. Analysis of the migration pattern of *C. pneumoniae* OMC proteins in SDS-PAGE. (A) *C. pneumoniae* OMC proteins were solubilized in SDS sample buffer. Then boiled (lane 1) and unboiled (lane 2) proteins were separated by SDS-PAGE and subjected to immunoblotting with MAb 24.1.44. (B) Silver staining of SDS-PAGE-separated *C. pneumoniae* OMC proteins solubilized in SDS sample buffer without boiling (lane 1). The bands marked with a vertical bar were excised from a Cu^{2+}-stained gel, boiled in SDS sample buffer, rerun in SDS-PAGE, and silver stained (lane 2). (C) Alternatively, the bands marked with the bar in panel B were excised, boiled and subjected to immunoblotting with PAb_{Omp4} (lane 1) and MAb 24.1.44 (lane 2).

in size to Omp4 was seen (Fig. 7A, lane 1). A reaction with a 40-kDa band was also seen. To determine whether this band was MOMP, we reacted recombinant MOMP in immunoblots with MAb 24.1.44 and a MAb (of the same Ig class) against *Mycoplasma hominis* PG21. Both MAbs gave a weak reaction with MOMP. This was also the case when we performed an immunoblot analysis in which the primary antibody was omitted (results not shown). A nonspecific reaction with MOMP has previously been noticed in immunoblot experiments (33). Thus, even though the MAb 24.1.44 recognized the 99-kDa band and MOMP with the same intensity, only the reaction with the 99-kDa band is specific.

When the MAb was reacted with *C. pneumoniae* OMC proteins separated by SDS-PAGE without boiling, two bands of 65 and 75 kDa were detected (Fig. 7A, lane 2). The migration of Omp4 was thus similar to that seen in the ovine abortion strain of *C. psittaci*, where a 98-kDa OMP migrated as 66 kDa when the protein was not heated (28).

The migration pattern of unboiled *C. pneumoniae* OMC proteins was analyzed by silver staining of SDS-PAGE-separated proteins. Several protein bands of 65 to 75 kDa were seen (Fig. 7B, lane 1). To investigate whether these bands contained proteins which changed migration upon boiling, the bands were excised from a polyacrylamide gel of *C. pneumoniae* OMC proteins separated without prior boiling and visualized by negative staining (28). When the protein content of these bands was extracted, boiled, and reanalyzed, it produced only one band of 99 kDa (lane 2), similar to what was seen for the *C. psittaci* protein (28). Immunoblotting of the extracted and boiled protein sample showed that both PAb_{Omp4} (Fig. 7C, lane 1) and MAb 24.1.44 (lane 2) recognized the 99-kDa protein. Thus, Omp4 was contained within the excised bands of 65 to 75 kDa. Immunoblotting of the excised and reanalyzed protein was also performed with PAbs against *C. pneumoniae* Omp2 and MOMP, but no reaction with these antibodies was seen (results not shown). These results demonstrated that the excised protein bands contained Omp4 but not MOMP or Omp2. Thus, without complete denaturation, Omp4 migrated as bands corresponding to 65 to 75 kDa that, upon heating, changed to a band of 99 kDa.

Antibody response of mice experimentally infected with *C. pneumoniae*. To analyze the humoral immune response to *C. pneumoniae* in experimentally infected mice and to determine whether Omp4 and Omp5 were immunogenic in such infections, four mice were experimentally infected with purified *C. pneumoniae* EBs. Sera were obtained from the mice 14 days after infection. The sera were reacted in immunoblot analyses of SDS-PAGE-separated proteins from *C. pneumoniae* EBs (Fig. 8A and B). Mouse serum 1 reacted with a 54-kDa band (Fig. 8A, lane 1), and mouse sera 2 and 3 reacted strongly with a band corresponding in size to Omp2 (lanes 2 and 3). This is in agreement with the size of proteins previously described to be immunogenic in human *C. pneumoniae* infections (8, 20, 21). The four mouse sera reacted weakly with several bands, of which 98- and 40-kDa bands were seen in all four lanes. Without boiling of the samples, all four mouse sera reacted strongly with a broad band that migrated as 65 kDa (Fig. 8B) and mouse serum 1 reacted with two additional bands (lane 1). Mouse serum 2 (Fig. 8A and B, lanes 2) was used in immuno-

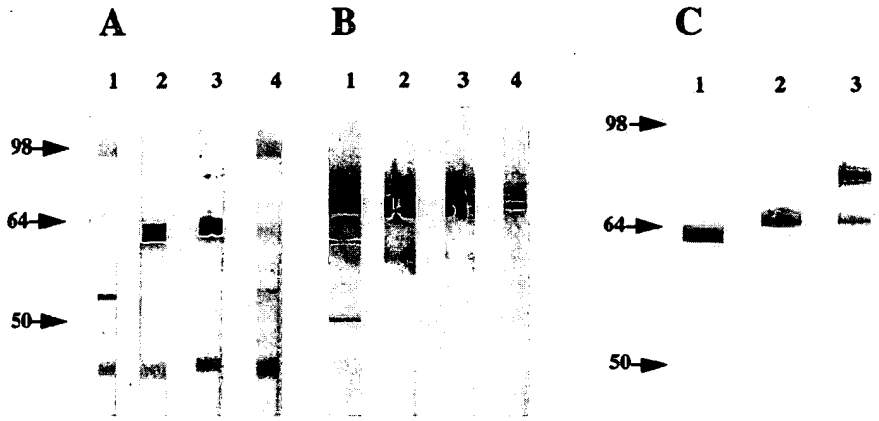

FIG. 8. Immunoblot analysis of four mouse sera obtained 14 days after nasal inoculation with 10^7 inclusion-forming units of *C. pneumoniae*. (A) *C. pneumoniae* EB proteins were solubilized in SDS sample buffer, boiled, separated by SDS-PAGE, and subjected to immunoblot analyses with the four sera. (B) *C. pneumoniae* EB proteins were suspended in SDS sample buffer without boiling and subjected to immunoblotting with the same four sera as used in panel A. (C) *C. pneumoniae* OMC proteins were solubilized in SDS sample buffer. The boiled (lane 1) and unboiled (lanes 2 and 3) proteins were subjected to immunoblotting. Lanes 1 and 2 were incubated with serum from mouse 2 (as in Fig. 8A and B, lanes 2), whereas lane 3 was incubated with PAb_{Omp4}.

blotting to analyze the reaction with *C. pneumoniae* OMC. Figure 8C (lane 1 [boiled] and 2 [unboiled]) showed the same reaction pattern as with the EB proteins. Thus, the immunogenic proteins are present in the *C. pneumoniae* OMC.

When the PAb_{Omp4} was reacted with unboiled *C. pneumoniae* OMC, two bands of 65 and 75 kDa were recognized (Fig. 8C, lanes 3). This result is similar to the immunoblotting results with MAb 24.1.44 (Fig. 7A, lane 2). Thus, the immunoblot analyses (Fig. 7 and 8) demonstrated that without boiling the Omp4 migrated as bands corresponding to sizes between 65 and 75 kDa, demonstrating incomplete denaturation of the unboiled proteins that results in a different conformation of Omp4. In addition, the results indicate that in experimentally infected mice, linear epitopes of Omp2 and conformational epitopes of Omp4 are dominant.

DISCUSSION

Components of the envelope of *Chlamydia* are important for attachment of the EBs to the host cell membrane and their subsequent uptake. Moreover, the outer membrane protects *Chlamydia* EBs in the extracellular environment against inactivation by antibodies. Of the three OMPs known to be present in COMC, Omp2 and Omp3 are not believed to be surface exposed (13). In contrast to MOMP from *C. trachomatis* and *C. psittaci*, the *C. pneumoniae* MOMP is genetically stable (14), is not a major immunogen (7), and is probably not surface exposed. Additional components of approximately 98 kDa were known to be present in the *C. pneumoniae* envelope (7, 30), but not until this study have the genes encoding proteins of this size been identified. Since previous studies indicated that antigenic reactivity is directed primarily against conformational epitopes of proteins at the *C. pneumoniae* surface (9, 34), we generated a polyclonal antiserum against SDS-denatured *C. pneumoniae* OMC and identified the genes encoding the two OMPs Omp4 and Omp5, of 98.9 and 97.2 kDa, respectively. Due to the content of cysteine residues in Omp4 (8 cysteines) and Omp5 (16 cysteines), these proteins probably correspond to the [^{35}S]cysteine-labeled OMPs of 98 kDa described by Melgosa et al. (30). We showed that Omp4 and Omp5 are located at the surface and that conformational epitopes of Omp4 seem to be dominant in experimentally infected mice. Therefore, this is the first study identifying genes encoding surface-exposed outer membrane proteins of *C. pneumoniae*.

Two additional expression clones encoding polypeptides with similarity to Omp4 and Omp5 were identified, and thus the genes constitute a gene family. Similar gene families exist in *C. psittaci* (27) and *C. trachomatis* (41), and a variable number of repeats of the amino acid motif Gly-Gly-Ala-Ile is a common feature of the deduced proteins. The repeated motif was not reflected at the DNA level, and therefore the origin of the gene family was probably not gene duplication. In general, the function of gene families in other pathogenic bacteria is to maintain antigenic variation in response to the host immune system. One example is the genes encoding the Opa proteins of *Neisseria gonorrhoea*, where phase variation generates switching among antigenically different proteins, allowing gonococci to avoid the host immune response (2). It is at present not known whether a similar mechanism by which EBs could switch the expression of the homologous OMPs and thus change their surface exists in *C. pneumoniae*.

Surface (S) layers have been detected on the outside of numerous gram-negative bacteria. In *Campylobacter fetus*, a family of S-layer proteins are encoded by multiple genes (12). The existence of silent genes in this gene family offers the possibility of varying the S layer. Thus, inversion of a chromosomal segment containing the promoter for a S-layer protein gene leads to the expression of another S-layer protein gene. By this mechanism the expression of the S-layer protein genes can be switched on and off, so that the *C. fetus* bacteria have the ability to vary both the antigenicity and structure of the S layer (12). The Omp4 and Omp5 gene family shows similarities to S-layer proteins, such as the amino acid composition and the requirement for boiling to permit complete denaturation. However, crystalline structure present at the surface of *C. pneumoniae* is required before the criteria for the proteins to be S-layer proteins can be fulfilled.

The *C. trachomatis* genes encoding PmpA to PmpI are located mainly in two clusters on the genome (41). Localization of related genes in clusters frequently indicates that they are important for pathogenicity, as described for the *fim* gene cluster from *E. coli* K-12 (22).

Despite the existence of similar gene families encoding surface-exposed proteins, our studies indicate that there are structural differences between the *C. pneumoniae* and *C. psittaci* envelopes. By immunoelectron microscopy, Longbottom et al. showed that proteins of the OMP90 family were exposed on the surface of both EBs and RBs (26), while the 80- to 90-kDa proteins from *C. psittaci* serotype 1 AB7 described by Buendia et al. (5) were exposed only on the surface of RBs. IMF with mouse PAbs indicated that *C. pneumoniae* Omp4 and Omp5 were located on the surface of the microorganism. However, these studies did not enable us to differentiate whether EBs, RBs, or both reacted. Use of immunoelectron microscopy, however, showed that epitopes of MAb 24.1.44, which reacted with recombinant Omp4, were located on the surface of EBs, RBs, and OMC.

In *C. psittaci* and *C. trachomatis*, MOMP is a major surface-exposed immunogen, but in *C. psittaci* the proteins of 90 to 98 kDa are also major antigens recognized by postabortion sera from ewes experimentally infected with *C. psittaci* (27). Even though a 98-kDa protein has been found to be immunogenic in *C. pneumoniae* infections (8, 21), a protein of this size was not a major immunogen when patient sera were reacted with purified *C. pneumoniae* EBs in immunoblot analyses (20). This is in agreement with our findings, since immunoblotting with sera from experimentally infected mice showed that the 97- to 99-kDa proteins were immunogenic only when the proteins were not fully denatured and thus migrated as 65 kDa. This is similar to the *C. psittaci* 98-kDa OMP, which migrates as 66 kDa in a SDS-PAGE when the protein is not heated; also, the 66-kDa band was shown to be immunogenic when reacted with pooled postabortion sheep sera (28).

Thus, conformational epitopes of Omp4 and possibly the other OMPs encoded by the *C. pneumoniae* gene family are likely to be the target for the humoral immune response in *C. pneumoniae* infections. The identification of this novel gene family may therefore facilitate the determination of the pathogenicity of this microorganism.

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Proteome analysis of the *Chlamydia pneumoniae* elementary body

Chlamydia pneumoniae is an obligate intracellular human pathogen that causes acute and chronic respiratory tract diseases and that has been implicated as a possible risk factor in the development of atherosclerotic heart disease. *C. pneumoniae* cultivated in Hep-2 cells were ^{35}S-labeled and infectious elementary bodies (EB) were purified. The EB proteins were separated by two-dimensional gel electrophoresis. Excised protein spots were in-gel digested with trypsin and peptides were concentrated on reverse-phase chromatographic beads for identification analysis by matrix-assisted laser desorption/ionization-mass spectrometry. In the pH range from 3–11, 263 *C. pneumoniae* protein spots encoded from 167 genes were identified. These genes constitute 15% of the genome. The identified proteins include 31 hypothetical proteins. It has recently been suggested that EB should be able to synthesize ATP. This view may be strengthened by the identification of several proteins involved in energy metabolism. Furthermore, proteins have been found which are involved in the type III secretion apparatus important for pathogenesis of intracellular bacteria. Proteome maps and a table of all identified proteins have been made available on the world wide web at www.gram.au.dk.

Keywords: *Chlamydia pneumoniae* / Two-dimensional electrophoresis / Matrix assisted laser desorption/ionization-mass spectrometry / Proteome / Make2Ddb EL 4289

1 Introduction

The genus *Chlamydia* consists of four species, *C. trachomatis, C. psittaci, C. pecorum,* and *C. pneumoniae. C. psittaci* and *C. pecorum* are animal pathogens and *C. psittaci* can cause ornithosis in humans. *C. trachomatis* and *C. pneumoniae* are human pathogens. Different biovars of *C. trachomatis* are the causative agents of sexually transmitted disease, trachoma and lymphogranuloma venereum. *C. pneumoniae* infects epithelial cells of the upper respiratory tract causing pneumonia and bronchitis [1]. Besides, *C. pneumoniae* has been suggested to play a role in atherogenesis by vascular infection. An association has been observed in sero-epidemiological studies [2], and *C. pneumoniae* has been isolated from atherosclerotic plaques [3], but no causality has been proved. Most likely everyone is infected with *C. pneumoniae* at least once during their lifetime. Reinfections are frequent and infections may become chronic. For review see Grayston [4]. The *Chlamydia* species are Gram-negative bacteria but only distantly related to other bacteria. All *Chlamydia* species are obligate intracellular parasites sharing a unique biphasic developmental cycle. The infective form, the elementary bodies (EB), attach to the host cell and are taken up by endocytosis. Inside a vacuole in the cytoplasm of the host cell the EB differentiate into reticulate bodies (RB) which utilize host cell nutrients for growth by binary fission before they reorganize into EB. A new generation of EB is released upon disruption of the host cell 48–84 h post infection (hpi). For general review see Moulder [5] and for analysis of structural development of *C. pneumoniae* see Wolf *et al.* [6].

C. pneumoniae was only established as a species in 1989 [7], and thus most work has been carried out on *C. trachomatis*. The outer membrane complex of *C. trachomatis* EB is osmotically stable, heavily cross-linked by disulfide bridges and hence very rigid. The DNA of *C. trachomatis* EB is packed into a condensed nucleoid structure by two histone-like proteins, Hc1 and Hc2 [8]. The genome sequence has been published for both *C. trachomatis* [9] and two different isolates of *C. pneumoniae* [10, 11]. The two *C. pneumoniae* isolates showed 99.9% identity [11], and 80% of the predicted coding sequences in *C. pneumoniae* had an ortholog in *C. trachomatis*. These orthologs showed an average of 62% identity on amino acid

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Abbreviations: CGP, *Chlamydia* Genome Project; **DTE**, dithioerythritol; **EB**, elementary body; **hpi**, hours post infection; **NL**, nonlinear; **OD**, optical density; **RB**, reticulate body

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level [10]. The size of the *C. pneumoniae* genome was found to be 1.2 kbp and 1073 ORFs were predicted [10].

For long it was believed that *Chlamydia* were energy parasites strictly auxotrophic for ATP, but the genome sequences revealed several energy producing enzymes. Genome sequences do not reveal which proteins are expressed or when they are present, and the developmental cycle of *Chlamydia* is only poorly understood. A better understanding of the protein content of EB will be of importance for elucidating the events that trigger differentiation of EB to RB and *vice versa*. The combination of two-dimensional gel electrophoresis for separation of proteins and MALDI-TOF-MS for identification has made it possible to perform functional genomics on a large scale. With the purpose of providing a proteome reference map for *C. pneumoniae* we have initiated a proteomics work on radiolabeled EB. Contrary to the RB, the EB can be highly enriched from infected host cells improving the results of MS. Unlike most staining procedures, radiolabeling does not introduce modifications of proteins interfering with MS. Furthermore, radiolabeling has the advantage that the developmental cycle of *Chlamydia* by means of pulse-labeling can be followed in time on a protein level without the necessity of purification of bacterial proteins. The pH range from 3–11 was investigated by using commercially available immobilized pH gradients and SDS PAGE 250–6 kDa. Theoretically 98% of the proteins possibly encoded by the predicted ORFs of the *C. pneumoniae* genome are within the investigated range. Only 25 proteins contain no methionines or cysteines except from the start-methionine.

2 Materials and methods

2.1 Organisms and cultivation

Hep-2 cells (ATCC, Rockville, MD, USA) were cultivated in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) added 10% fetal calf serum (Gibco BRL; heat-inactivated and sterile filtered), 10 mg/L gentamycine and 0.3 g/L glutamine at 37°C in a 5% CO_2 and 85% humidity atmosphere. Semiconfluent monolayers of cells in 6-well trays (TPP, Switzerland) (3.5 cm well diameter) were infected with one inclusion forming unit per cell of *C. pneumoniae* (VR 1310, ATTC) by 30 min of centrifugation at 2400 rpm in a Beckmann GS-6R centrifuge. Infected cells were cultivated at 34°C in Hep-2 media with addition of 2 mg/L gentamycine, 1 μg/mL cycloheximide and 0.5% glucose.

2.2 ^{35}S-labelling

Labeling was performed in two wells for 2 h periods at 6, 12, 24, 36, 42, 48, 54, and 60 hpi in a methionine/cysteine-free RPMI 1640 medium containing 40 μg/mL cycloheximide and 100 μCi/mL [^{35}S]methionine/cysteine (70:30) (Promix; Amersham, Buckinghamshire, England).

2.3 Sample preparation

Infected cells were scraped off in 1 mL PBS per well. Labeled samples from 16 wells were mixed with unlabeled samples from 42 wells and purification of EB was performed by ultracentrifugation essentially as previously described [12] except that Urografin was replaced by Visipaque (Nycomed, Oslo, Norway). Protein amount was determined using a bicinchoninic acid assay system (Pierce, Rockford, IL, USA). EB samples were dissolved in 1% SDS, 50 mM Tris-HCl, pH 7.0, sonicated and boiled for 5 min. After cooling, a lysis solution was added containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM Tris-base, 65 mM dithioerythritol (DTE) and 2% v/v Pharmalyte pH 3–10 (Amersham Pharmacia Biotech, Uppsala, Sweden). This procedure is essentially as described by Harder *et al.* [13]. The amount of incorporation was determined by TCA precipitation and scintillation counting.

2.4 Two-dimensional gel electrophoresis

For isoelectric focusing, 18 cm immobilized pH gradient drystrips (Pharmacia, Uppsala, Sweden) were used with pH ranges of 4–7, 3–10 nonlinear (NL) and 6–11. Strips for preparative gels were rehydrated overnight in 380 μL of the lysis solution containing 3 000 000 cpm and adjusted to 750 μg of protein by addition of unlabeled EB protein. Rehydrated strips were run on a Multiphor II (Pharmacia) at 20°C for 1 h at 250 V, 1 h at 300 V, 1 h at 400 V, 2 h at 500 V, 2 h at 1000 V, 3 h at 3500 V and 48 h at 5000 V. After the first dimension, strips were equilibrated in 6 M urea, 2% SDS, 30% glycerol (87%) and 2% DTE using 2.5 mL per strip in a reswelling tray gently shaking for 15 min and subsequently for 15 min in a buffer where iodoacetamide replaced DTE. Linear gradient polyacrylamide gels for second dimension were cast by use of a gradient maker. Light solution: 9% T, 0.2% SDS, 375 mM Tris-HCl, pH 8.8. Heavy solution: same but 16% T, 20% glycerol. The cross-linking agent was methylene-bisacrylamide (2.6% C). Solutions were filtered and degassed before 332 μL of 10% APS and 254 μL of 100% TEMED per 100 mL of each solution were added and mixed. Gels were overlaid with 10% ethanol and polymerized for 4 h before use. Separation was performed at 120–150 V until the dye front had reached the bottom of the gels. Preparative gels for MS were washed for 30 min in double-distilled water and vacuum-dried on a paper backing before exposure to Kodak Biomax-MR X-ray films. For comparative gels only 300 000 cpm was loaded per strip,

the final focusing step was reduced to 24 h and gels were fixed in a solution containing 10% acetic acid and 25% 2-propanol for 30 min and treated with Amplify (Amersham) for another 30 min before drying. All gels were exposed to X-ray films for seven days at –70°C.

2.5 Protein and peptide preparation procedures

The excised protein spots were briefly submerged in water (Baker HPLC Analyzed; Mallinckrodt Baker B.V., Deventer, The Netherlands), and the paper backing was removed from the reswollen gel pieces. Subsequently, the gel pieces were washed twice for 15 min in 100 μL of acetonitrile/water (1:1) (both Baker HPLC Analyzed from Mallinckrodt Baker B.V.) and dried in a centrifugal vacuum concentrator. The dried gel pieces were rehydrated in 10 μL of 50 mM freshly prepared ammonium bicarbonate buffer containing 0.05 μg of sequencing grade modified trypsin (Promega, Madison, WI, USA). Ammonium bicarbonate buffer was added so as to completely submerge the gel pieces (typically 50–100 μL) and digestion was allowed to proceed overnight at 37°C. Following digestion, the supernatant containing the generated peptides was removed from the gel pieces and acidified by adding 5 μL of 10% trifluoroacetic acid in water. The obtained peptide mixtures were purified and concentrated on a small amount of added Poros® 50 R2 beads (Boehringer Mannheim GmbH, Mannheim, Germany) and stored at –20°C or immediately used for MALDI-MS analysis as described [14, 15].

2.6 MALDI-TOF-MS peptide mass fingerprinting

All MALDI-MS measurements were performed on a Bruker Reflex III MALDI-TOF-MS (Bruker Daltonik GmbH, Bremen, Germany) operating in reflectron mode (for details of operation see Gevaert *et al.* [15]). Prior to each peptide mass fingerprint analysis the instrument was externally calibrated using the known masses of a mixture of synthetic peptides, spotted on the target disc as close as possible to the real sample. Typically 50–100 laser shots were accumulated during each measurement. The peptide masses were exported to the PeptideSearch algorithm [16] to identify proteins in a locally stored database containing only the *C. pneumoniae* proteins (downloaded from http://www.ncbi.nlm.nih.gov/Entrez/batch.html). As for peptide mass fingerprinting, peptide mass accuracies better than 100 ppm were considered in the database searches.

2.7 Computer analysis

Autoradiographies were scanned at a resolution of 306 dpi, and images were annotated in Melanie II for SUN Solaris (BioRad, Hercules, CA, USA), which was also used for quantification of spots. The software used for construction of the database was the Make2Ddb for SUN Solaris from the ExPASy server (http://www.expasy.ch/ch2d/make2ddb.html). Computation of p*I*/M_r for identified proteins was performed by use of the Compute p*I*/M_r tool at the ExPASy server (http://www.expasy.ch/tools/pi_tool.html). To obtain approximate values of p*I*/M_r for all *C. pneumoniae* proteins, the TagIdent tool at the ExPASy server was used. Other protein sequence analysis was carried out by the use of programs from the Wisconsin Package (GCG) located at www.biobase.dk. Signal peptidase I cleavage sites were predicted by comparison to the ProSite PS00013 pattern. Signal peptidase II cleavage sites were predicted by the Signal-P server V2.0.b2 (http://www.cbs.dtu.dk/services/SignalP-2.0/).

3 Results and discussion

3.1 Resolution of 2-D images

To avoid modifications caused by staining procedures and to benefit from the advantages of pulse labeling in further analysis, radiography was chosen for visualization of proteins. To achieve incorporation in proteins synthesized at different times during the developmental cycle, labeling was performed at several timepoints throughout the cultivation period. Purified EB were boiled in SDS and thiourea was included in the lysis buffer to improve solubilization of outer membrane proteins. Proteins were separated by 2-D PAGE using IPG gradients of pH 4–7 (IPG 4–7), pH 6–11 (IPG 6–11) and nonlinear pH 3–10 (NL IPG 3–10). For analysis by MS the protein load was 3 000 000 cpm and 750 μg per gel and spots were pooled from two preparative gels. For comparative gels which were used for annotation and quantification of identified spots the protein load was 300 000 cpm. Films were exposed to preparative gels to reveal as many proteins as possible whereas exposure to comparative gels was shortened to avoid overexposure of areas with highly abundant proteins. Comparative gels are presented in Fig. 1.

Despite difference in exposure, it was possible to match identified spots from the preparative gels to the comparative images in all except seven cases for which the positions of the proteins were marked by the use of nearby landmarks. In Fig. 2 identifications are mapped to a region from the IPG 4–7 image. With an exposure time of one week for the comparative gels, 424 protein spots were resolved in the range from pH 4.0–6.0 using IPG 4–7 (Fig. 1a), 155 proteins spots were resolved in the range from pH 6.0–11.0 using IPG 6–11 (Fig. 1b) and 554 protein

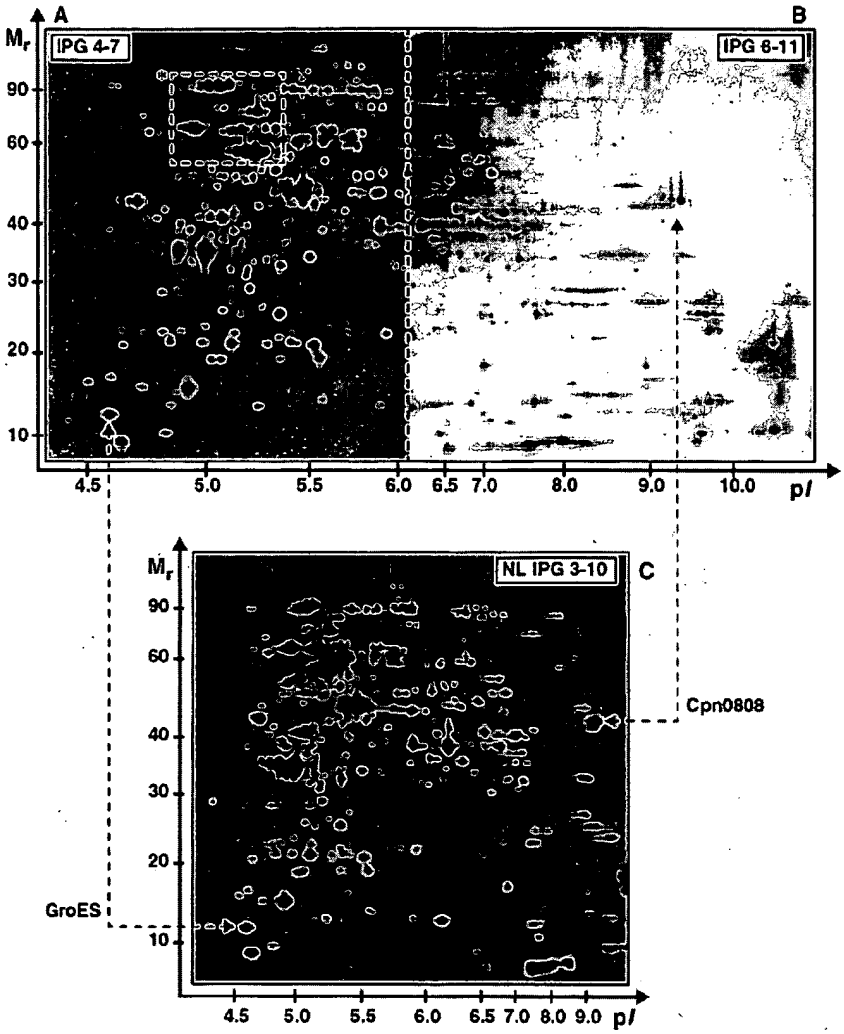

Figure 1. Images of comparative gels. Gel load was 300 000 cpm and the exposure time was seven days. Images of (A) IPG 4–7 and (B) IPG 6–11 are overlaid based on a pattern of nine spots (of which five were identified) in the pH range from 6.0–6.2. (C) Arrows indicate spots matched to the NL IPG 3–10 image. * The boxed detail is enlarged in Fig. 2 where identified spots are labeled.

spots were resolved in the range from 4.0–9.5 using NL IPG 3–10 (Fig. 1c). The observed distribution of spots with respect to pH for all gels is illustrated in Fig. 3. An overlapping area from pH 6.0–6.2 between IPG 4–7 and IPG 6–11 was determined by five identified proteins and an easily recognizable pattern of nine spots. However, the pH range from 6–7 was best resolved using NL IPG 3–10 (115 resolved spots *versus* 54 for IPG 6–11). Combining pH ranges 4–6 from IPG 4–7, 6–7 from NL IPG 3–10 and 7–11 from IPG 6–11, a total of 640 spots were resolved.

In Fig. 4 the observed number of spots in pH intervals of one are compared to the theoretical number of proteins in each range as predicted by running the program TagIdent on *C. pneumoniae* proteins present in the SWISS-PROT and TrEMBL databases. The whole pH range from 4–11 was predicted to contain 1051 proteins or 98% of 1079 *C. pneumoniae* proteins present in these databases. The theoretical distribution of proteins peaks at the intervals 5–6 and 9–10. The acidic range holds most enzymes and structural proteins whereas the basic range holds most nucleic acid binding proteins. Clearly most spots were observed in the acidic area. Below pH 6 the number of observed spots exceeded the number of predicted proteins by 5% but above pH 6 the ratio of detected spots to predicted proteins was below 0.30 except for the range from p*I* 7–8 where the ratio was 0.56.

The resolution in 2-D PAGE is generally reported to be lower for basic than for acidic proteins as, for instance, in the *M. genitalium* proteome [17]. It can be speculated that

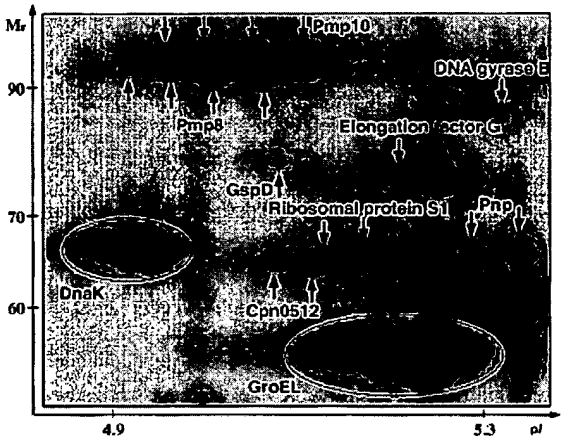

Figure 2. Detail from the IPG 4–7 image (Fig. 1). Identified spots are encircled or marked with arrows.

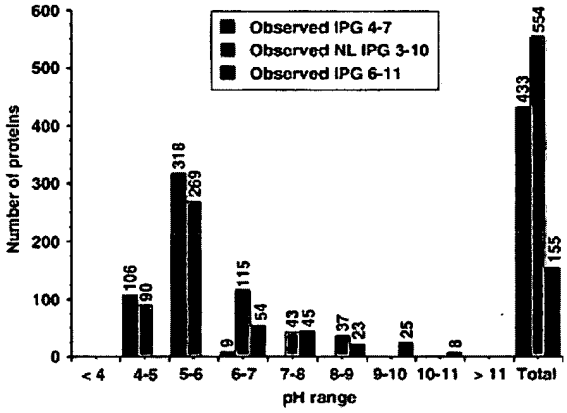

Figure 3. Distribution of spots observed in each of the investigated pH ranges of 4–7, NL 3–10 and 6–11. 300 000 cpm was loaded in each case and autoradiographies were analyzed with the Melanie II software after seven days of exposure.

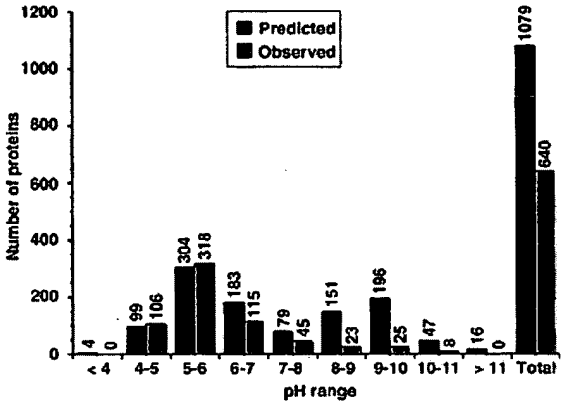

Figure 4. Theoretical distribution of proteins in pH intervals of one compared to the observed spot distribution. The theoretical distribution was predicted by use of the TagIdent tool at the ExPASy server on all *C. pneumoniae* proteins present in the SWISS-PROT and TrEMBL databases. The numbers of observed spots are from the image showing best resolution in each pH interval: 4–6 is from the IPG 4–7, 6–7 is from the NL IPG 3–10 and 8–11 is from the IPG 6–11.

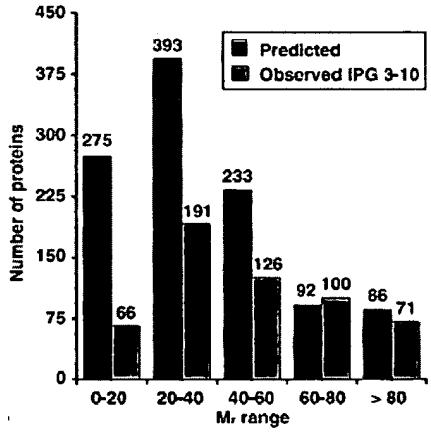

Figure 5. Theoretical distribution of proteins in M_r intervals of 20 kDa compared to the observed spot distribution. The theoretical distribution was predicted by use of the TagIdent tool at the ExPASy server on all *C. pneumoniae* proteins present in the SWISS-PROT and TrEMBL databases. The observed distribution is deduced from the NL IPG 3–10 image.

the highest coverage from pH 4–6 in this study is due to superiority of the IPG 4–7 strip, but there can be many causes for lack of spots. It must be noted that the predicted protein distribution includes the whole genome whereas we only observe on the subpopulation of proteins present in EB. Many basic DNA binding proteins will only be present in low amounts in general and maybe in EB in particular. In general, the expression level must be high enough for detection and identification, and the turnover not too fast. Furthermore, proteins must be solubilized and transferred to the SDS-gel which in particular is a problem for highly hydrophobic proteins. Finally, ^{35}S-labeling requires content of methionine or cysteine for proteins to be visualized. In Fig. 5 distribution with respect to M_r ranges in the NL IPG 3–10 image is illustrated. If it is

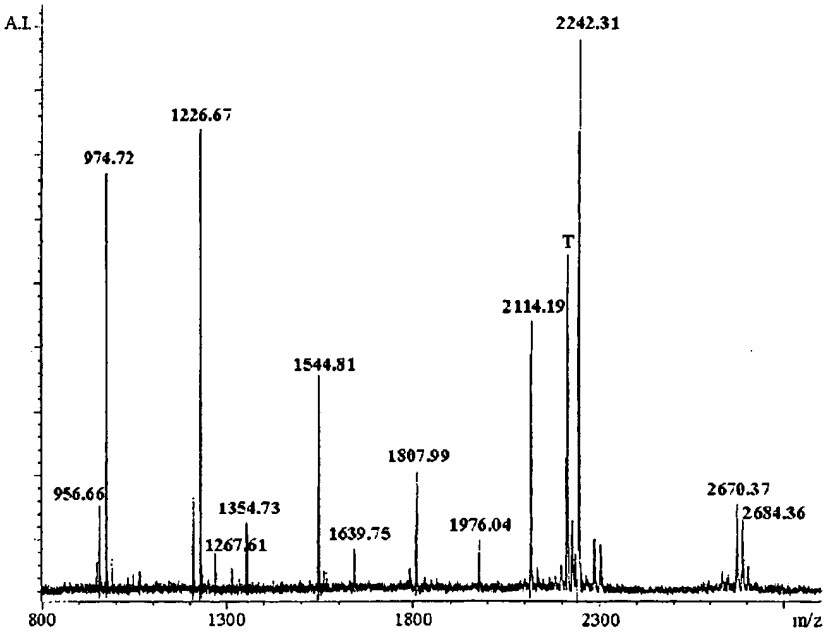

Figure 6. MALDI-TOF-MS tryptic peptide map of spot number 253. Peptides originating from the identified protein, Cpn0917 are indicated. T denotes autodigestion product of trypsin. Observed p*I*/M_r of spot 253 : 5.10/18894. Predicted p*I*/M_r of Cpn0917:5.13/17410.

taken into consideration that more isoforms are present in the high M_r area than in the low M_r area, the best coverage is obtained in the range from 20–40 kDa.

As seen in Fig. 1, the most acidic spots detected with the use of IPG 4–7 were included in the NL IPG 3–10 image and only about 20 spots present at a p*I* higher than 9.5 were resolved with the use of IPG 6–11 and not NL IPG 3–10. The overall resolution of the NL IPG 3–10 was not as good as for the narrower gradients, except for the pH range from 6–7, but only very few spots could not be matched between gel images due to lack of resolution power of this strip. The fact that most spots can be matched between the narrow gradients 4–7 and 6–11 and the NL EPG 3–10 reduces the effort of further analysis since the number of gels that have to be examined is limited.

3.2 Identification of proteins

A total of 351 protein spots separated by the use of IPG 4–7 and 6–11 were excised from two replicative gels and in-gel digested with trypsin. Peptides were concentrated on chromatographic beads and analyzed by MALDI-TOF-MS. The MS spectra resulting in the identification of Cpn0917 is given as an example in Fig. 6. From the 351 analyzed samples 236 *C. pneumoniae* proteins were identified. Twenty-three samples led solely to the identification of human proteins with actin, alpha-tubulin and beta-tubulin being the most abundant. Fourty-three contained too little protein material and a few were contaminated by keratines. The rest led to unambiguous identifications. When the same protein was identified in two or more samples from a train of spots clearly arising from isoforms of the same protein, the identification was extended to cover all spots in this row. Fourteen spots were identified this way resulting in a total of 250 identified spots. From these 243 could be matched to the NL IPG 3–10 image by pattern recognition. Correct match was confirmed for all of 27 proteins also identified from the NL IPG 3–10. In addition, thirteen novel identifications were obtained from the NL IPG 3–10. All together, 263 different proteins spots were identified and 167 of these were encoded by distinct genes covering 15% of the predicted ORFs encoded by the genome. Isoforms of different p*I* were detected for 61 proteins.

Extrapolating the obtained results, a total of 640 resolved protein spots can be expected to contain bacterial protein in 583 cases (640 × 236/[236 + 23]). If the percentage of isoforms calculated from identified protein spots holds true these 583 proteins represent 370 genes (583 × 167/263). The coding capacity of *C. pneumoniae* in the range from pH 3–11 is 1051 proteins but only a fraction of these can be expected to be present in EB as some proteins must be RB-specific and some only expressed under certain growth conditions. The solubilization procedure used in this study was optimized for recovery of outer mem-

brane proteins and many of these were in fact identified. To increase the total number of identifications, a set of different solubilizing agents could be used. Narrowing the pH gradients would probably only result in few more detections considering the low number of proteins and the good separation observed.

3.3 Quantification of protein spots

Protein spots from the NL IPG 3–10 image were quantified by integrating optical density (OD) over Gaussian area using the Melanie II software. From the 100 protein spots of highest volume 84 were identified and 79% of the total volume in this gel was accounted for. The 15 spots of highest volume were identified as listed in Table 1. A number for the approximate molar amount of each identified protein was obtained by adding spot volumes for all identified isoforms of a given protein and correcting for the number of methionines and cysteines present after cleavage of predicted leader peptides. The molar amount was converted to percentage of total protein amount in the gel extrapolating results from identified spots to unidentified. The 16 identified proteins each representing more than 1% of the total protein amount in the gel are listed in Table 2. These include four hypothetical proteins (Cpn0720, Cpn1032, Cpn0808 and Cpn0677), the three stress proteins DnaK, GroEL and GroES, ribosomal proteins, Clp1 and Clp2, two Pmps, Omp2 and MOMP. The by far most abundant protein in the gel was MOMP representing 20%.

It must be emphasized that the calculated percentages are only estimates and do not reflect the actual amount of proteins in EB but in the gel. Furthermore, the exact incorporation rate of radioactivity is not known. A mixture of methionine and cysteine was used to achieve incorporation in proteins lacking one of these amino acids. Quantification was carried out for the NL IPG 3–10 image since this contained the highest number of spots.

3.4 Identified proteins

The identified proteins were categorized according to the *Chlamydia* Genome Project (CGP) [18] in which functional assignments have been based upon similarity to genes of known function from other organisms, mainly *Escherichia coli*. Our findings are presented in Table 3 and commented on in the following. Subheadings almost strictly refer to CGP categories.

3.4.1 Protein folding, assembly, and modification

Chaperones mediate protein folding. Some of these proteins are also known as heat shock proteins because they are upregulated by heat shock. Chaperones are well con-

Table 1. The 15 spots of highest volume in the NL IPG 3-10 image

Accession	Protein	Volume	Volume %
Q9Z9A7	EF-Tu	12.7	4.5
P27455	MOMP	12.3	4.3
P31681	GroEL	7.5	2.6
P27455	MOMP	6.7	2.4
P27455	MOMP	6.6	2.3
Q9Z798	Cpn0808	5.3	1.9
Q9Z9A1	RL-7	5.0	1.7
Q9Z9A7	EF-Tu	4.7	1.7
P23700	Omp2	4.2	1.5
P27455	MOMP	4.0	1.4
P317A6	Enolase	3.9	1.4
Q9Z7S8	RNA polymerase A	3.7	1.3
Q9Z7P2	AsrS/Omp2	3.5	1.2
P31681	GroEL	3.4	1.2
P31682	GroES	3.4	1.2

The autoradiography was scanned and analyzed with the Melanie II Software. Spot volume was calculated as OD integrated over Gaussian area.

Table 2. Identified proteins representing more than 1.0% of total spot volume in the NL IPG 3-10 image

Accession	Protein	Vol. %
P27455	MOMP	20.5
Q9Z9A1	RL7	7.4
Q9Z9A7	EF-Tu	4.8
P31682	GroES	3.3
P31681	GroEL	2.9
Q9Z7I5	Cpn0720	2.0
Q9Z6M7	Cpn1032	1.6
P27542	DnaK	1.4
Q9Z7S8	RNA polymerase A	1.3
Q9Z759	Clp2	1.3
Q9Z798	Cpn0808	1.2
Q9Z832	Clp1	1.2
O86163	Pmp10	1.1
P23700	Omp2	1.1
Q9Z7M7	Cpn0677	1.1
Q9Z393	Pmp8	1.0

Spot volumes were added for all identified isoforms of each protein and corrected for number of Met/Cys.

Table 3. List of C. *pneumoniae* proteins identified from purified EB

Category according to the CGP	Protein name (SW/Tr)	Gene	Nb[a)]	Acc.[b)]	Theor. p*I*/M_r[c)]	Obs. p*I*/M_r[d)]	P/L[e)]
Amino acid biosynthesis:							
Base and nucleotide metabolism	GMP kinase	*gmk*	0120	Q9Z961	5.73/23368	5.74/22251	
Base and nucleotide metabolism	Thioredoxin reductase (TRXR)	*trxB*	0314	Q9Z8M4	6.24/33559	6.16/32565	
Base and nucleotide metabolism	CMP kinase	*cmk*	0568	Q9Z7Y5	5.62/24080	5.61/22972	
Base and nucleotide metabolism	Uridine 5'-mono-phosphate synthase	*pyrF*	0608	Q9Z7U6	6.61/22895	6.59/21607*	
Base and nucleotide metabolism	Thioredoxin (TRX)	*trxA*	0659	Q9Z7P5	4.88/11292	4.80/10209	
Base and nucleotide metabolism	UMP kinase (UK)	*pyrH*	0698	Q9Z7K7	5.39/27098	5.18/24847 5.35/24847	
Base and nucleotide metabolism	AMP nucleosidase	*amn*	0894	Q9Z712	6.56/32663	6.53/30651* 6.74/30623*	
Base and nucleotide metabolism	Cytosine deaminase	*yfhC*	1001	Q9Z6Q8	5.74/17440	5.75/14099	
Biosynthesis of cofactors:							
Folic acid	Methylene tetra-hydrofolate dehydro-genase	*folD*	0335	Q9Z8K3	7.08/30632	7.32/30853*	
Folic acid	Probable folate synthesis protein	*folP*	0758	Q9Z7E8	5.39/49815	5.36/47139	
Riboflavin	Riboflavin synthase beta-chain	*ribE*	0873	Q9Z733	7.69/16304	7.66/12341*	P
Cell envelope:							
Fatty acid and phos-pholipid metabolism	Biotin carboxyl carrier protein	*accB*	0183	Q9Z901	5.05/18461	4.81/21186 4.81/21712	
Fatty acid and phos-pholipid metabolism	Lysophospholipase esterase	*CPN0271*	0271	Q9Z8R7	5.09/27221	5.18/23629	
Fatty acid and phos-pholipid metabolism	3-Oxoacyl-(acyl-carrier protein) reductase	*fabG*	0296	Q9Z8P2	7.69/26505	7.69/23580*	
Fatty acid and phos-pholipid metabolism	Malonyl acyl carrier transcyclase	*fabD*	0297	Q9Z8P1	5.17/33845	5.14/30900 5.21/30867	
Fatty acid and phos-pholipid metabolism	Enoyl-acyl-carrier protein reductase	*fabI*	0406	Q9Z8D7	5.50/31989	5.33/33257 5.50/33257	
Fatty acid and phos-pholipid metabolism	Acyl carrier protein synthase	*fabF*	0916	Q9Z6Z0	5.52/44695	5.41/43359 5.58/43359 5.67/43477	
Fatty acid and phos-pholipid metabolism	Oxoacyl carrier protein synthase III	*fabH*	0298	Q9Z8P0	5.44/36081	5.29/36446 5.39/36486	
Fatty acid and phos-pholipid metabolism	ACCOA carboxylase/ transferase alpha	*accA*	0414	Q9Z8C9	6.23/36347	6.29/34272	
LPS	CMP-KDO synthetase (CKS)	*kdsB*	0235	Q9Z8U9	5.70/28414	5.71/27691	
LPS	UDP glucosamine *N*-acyltransferase	*lpxD*	0302	Q9Z8N6	7.86/38847	8.01/38068*	?
LPS	Myristoryl GlcNac dea-cetylase	*lpxC*	0652	Q9Z7Q2	5.99/31036	5.99/30985	

Table 3. Continued

Category according to the CGP	Protein name (SW/Tr)	Gene	Nb[a)]	Acc.[b)]	Theor. p*I*/M_r[c)]	Obs. p*I*/M_r[d)]	P/L[e)]
Membrane proteins, lipoproteins, porins	Omp85 analog	*yaeT*	0300	Q9Z8N8	8.28/88957	7.33/73942 7.52/74068 #	P
Membrane proteins, lipoproteins, porins	Omp-like outer membrane protein	*CPN0301*	0301	Q9Z8N7	4.98/19482	4.73/15253	P
Membrane proteins, lipoproteins, porins	Omp2	*omp2*	0557	P23700	6.01/59719	5.34/60278 5.37/61783 5.38/60651 5.39/67770 5.49/62454 5.69/62165 5.71/61974	P
Membrane proteins, lipoproteins, porins	MOMP	*momp*	0695	P27455	7.50/41620	4.84/34947 5.01/34478 5.36/31459	P
Peptidoglycan	MURC/DD bifunctional enzyme	*murC/ddlA*	0905	Q9Z701	5.57/89964	5.61/81586	
Cellular processes:							
Cell division	Chromosome partitioning protein	*parB*	0684	Q9Z7M0	6.11/32057	6.18/34634	
Cell division	Axial filament protein	*cafE*	0959	Q9Z6U9	6.32/59551	6.52/59926 *	
Cell division	Cell division protein FTSY	*ftsY*	0972	Q9Z6T7	6.97/31758	7.08/29335 *	
Detoxification	Superoxide dismutase	*sodM*	0057	Q9Z9C4	5.53/23541	5.18/20877 5.30/20928 5.44/21005	
Detoxification	Thio-specific antioxidant peroxidase	*ahpC*	0778	Q9Z7C8	4.83/21900	4.79/22471	
Signal transduction	PTS IIA protein + DNA-binding domain	*ptsN_2*	0061	Q9Z9C0	5.36/25860	5.35/21739	
Signal transduction	GTP binding protein	*ychF*	0321	Q9Z8L7	5.22/40058	5.21/39651	
Standard protein secretion	Signal recognition particle GTPase	*ffh*	0115	Q9Z966	8.90/50607	8.91/45513	(P)
Standard protein secretion	General secretion protein D	*gspD*	0815	Q9Z791	5.19/83521	5.11/78239	P
Standard protein secretion	Trigger factor-peptidyl-prolyl isomerase	*tigA*	0848	Q9Z758	5.05/49839	5.06/51186	?
Transport-related proteins	ABC transporter protein ATPase	*yjjK*	0023	Q9Z9F8	5.40/59110	5.34/55274 5.41/55130	
Transport-related proteins	ABC transporter membrane protein	*CPN0690*	0690	Q9Z7L4	4.97/45806	5.01/43085	P
Transport-related proteins	ABC transporter ATPase	*abcX*	0691	Q9Z7L3	5.22/28663	5.14/28654	
Type III secretion	Low calcium response E	*lcrE*	0324	Q9Z8L4	4.98/43406	4.80/42426	
Type III secretion	YOP C/general secretion protein D	*yscC*	0702	Q9Z7K3	6.02/100392	4.74/73051	P
Type III secretion	YSC N (ATPase)	*yscN*	0707	Q9Z7J8	5.84/48106	5.71/45580 5.78/41930 5.78/45507	
Type III secretion	YOP L	*yscL*	0826	Q9Z780	4.96/25848	4.79/28138	

Table 3. Continued

Category according to the CGP	Protein name (SW/Tr)	Gene	Nb[a)]	Acc.[b)]	Theor. pI/M_r[c)]	Obs. pI/M_r[d)]	P/L[e)]
Central intermediate metabolism:							
Glycogen metabolism	Glycogen phosphorylase	*glgP*	0307	Q9Z8N1	5.83/94517	5.76/78968 5.78/78239	
Glycogen metabolism	Phosphoenolpyruvate carboxykinase	*pckA*	0851	Q9Z755	5.55/66992	5.49/60185	
Phosphorous and sulfur	Inorganic pyrophosphatase	*ppa*	0918	Q9Z6Y8	5.01/23981	5.08/27151	
Chlamydia-specific proteins:							
Polymorphic outer membrane protein	Pmp2	*pmp_2*	0013	Q9Z3A1	6.02/89601	5.71/90162 5.75/88883	P
Polymorphic outer membrane protein	Pmp6	*pmp_6*	0444	Q9Z899	5.34/144920	4.61/95000 4.65/94488 4.66/94148	P
Polymorphic outer membrane protein	Pmp7	*pmp_7*	0445	Q9Z898	5.73/100080	5.38/91802 5.56/90162 5.61/90000	P
Polymorphic outer membrane protein	Pmp8	*pmp_8*	0446	Q9Z393	5.16/94794	4.92/9135 4.98/92633 5.05/92300 5.10/91968 5.13/92968	P
Polymorphic outer membrane protein	Pmp10	*pmp_10*	0449-0450	O06163	5.22/95604	4.97/96658 5.03/96868 5.09/96658 5.13/96658 5.17/96033	L
Polymorphic outer membrane protein	Pmp11	*pmp_11*	0451	O86164	5.98/98904	5.71/92134 5.73/91143 5.76/90488	L
Polymorphic outer membrane protein	Pmp13	*pmp_13*	0453	Q9Z896	6.55/100143	6.46/92613 6.54/92026 #	P
Polymorphic outer membrane protein	Pmp14	*pmp_14*	0454	Q9Z895	6.79/101185	6.67/87389 6.74/87217 #	P
Polymorphic outer membrane protein	Pmp20	*pmp_20*	0540	Q9RB59	5.40/179594	5.70/114176	P
Polymorphic outer membrane protein	Pmp21	*pmp_21*	0963	Q9RB58	4.91/170884	5.50/47026	P
DNA replication, recombination:							
DNA Replication	DNA gyrase subunit B	*gyrB_1*	0275	Q9Z8R3	5.33/90571	5.32/85308	
DNA Replication	DNA polymerase III beta-chain	*dnaN*	0338	Q9Z8K0	4.96/40334	4.91/38708	
DNA Replication	Single-strand binding protein (SSB)	*ssb*	0386	Q9Z8F7	5.41/17823	5.41/21160	
DNA Replication	Replicative DNA helicase	*dnaB*	0616	Q9Z7T8	5.94/51043	5.94/45253	
Recombination	RECA Protein	*recA*	0762	Q9z7e4	6.68/38228	6.64/38713 * 6.95/38677 *	

Table 3. Continued

Category according to the CGP	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Theor. p*I*/M_r[c]	Obs. p*I*/M_r[d]	P/L[e]
Energy metabolism:							
ATP biogenesis and metabolism	ATP synthase subunit B	*atpB*	0089	Q9Z992	5.69/48286	5.61/48208 5.72/48379	
Glycolysis and gluconeogenesis	Pyruvate kinase	*pyk*	0097	Q9Z984	5.36/52666	5.20/50788 5.27/50655 5.34/50655 5.41/50524	
Glycolysis and gluconeogenesis	Glyceraldehyde-3-phosphate dehydrogenase	*gapA*	0624	Q9Z7T0	6.25/36837	6.37/35459 *	
Glycolysis and gluconeogenesis	Phosphoglycerate kinase	*pgK*	0679	Q9Z7M5	6.07/43046	5.87/41364 * 6.11/41214 *	
Glycolysis and gluconeogenesis	Enolase	*eno*	0800	Q9Z7A6	4.66/46104	4.70/44152 4.72/44032	
Glycolysis and gluconeogenesis	Phosphoglycerate mutase	*pgmA*	0863	Q9Z743	7.83/26246	7.03/24882 * 7.83/25078 *	
Glycolysis and gluconeogenesis	Triosephosphate isomerase (TIM)	*tpiS*	1063	Q9Z6J6	5.04/27602	5.01/23142	
Pentose phosphate pathway	Glucose 6P dehydrogenase	*devB*	0239	Q9Z8U5	4.92/29333	4.79/28138	
Pentose phosphate pathway	Transketolase	*tktB_1*	0893	Q9Z713	5.73/73478	5.74/64013	
Pyruvate dehydrogenase	Pyruvate dehydrogenase alpha	*pdhA*	0304	Q9Z8N4	5.13/37466	5.10/36969	
Pyruvate dehydrogenase	Dihydrolipoamide acetyltransferase	*pdhC*	0306	Q9Z8N2	5.90/46256	5.75/48567	
Pyruvate dehydrogenase	Lipoate protein ligase-like protein	*lplA_1*	0436	Q9Z8A7	5.63/2667	5.70/23629	
Pyruvate dehydrogenase	Nucleoside-2-P kinase	*ndk*	0619	Q9Z7T5	5.34/15642	5.25/12933	
Pyruvate dehydrogenase	Lipoamide dehydrogenase	*lpdA*	0833	Q9Z773	6.19/49167	6.38/46446 *	
TCA cycle	Aspartate aminotransferase	*aspC*	0495	Q9Z856	5.48/44491	5.47/39651	
TCA cycle	Succinyl-CoA synthetase beta-chain	*sucC*	0973	Q9Z6T6	5.19/41925	5.15/39539 5.18/39825	
TCA cycle	Succinyl-CoA synthetase alpha-chain	*sucD*	0974	Q9Z6T5	5.19/30707	5.29/32761	
TCA cycle	Fumarate hydratase	*fumC*	1013	Q9Z6P6	5.79/50441	5.76/45289	
TCA cycle	Malate dehydrogenase	*mdhC*	1028	Q9Z6N1	7.16/35967	7.05/32117 * 7.47/32237 *	
Hypothetical proteins:							
Hypothetical protein	Hypothetical protein Ct017	*CPN0104*	0104	Q9Z977	6.48/44492	6.47/38677 *	P
Hypothetical protein	Hypothetical protein Ct031	*CPN0121*	0121	Q9Z960	9.43/11098	9.53/10913 *	
Hypothetical protein	Hypothetical protein	*yxjG_1*	0143	Q9Z940	5.50/42846	5.41/39263	
Hypothetical protein	Hypothetical protein Ct273	*CPN0422*	0422	Q9Z8C1	5.01/20863	5.29/21980	
Hypothetical protein	Hypothetical protein Ct386	*CPN0489*	0489	Q9Z862	5.40/33474	5.44/29198	

Table 3. Continued

Category according to the CGP	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Theor. pI/M_r[c]	Obs. pI/M_r[d]	P/L[e]
Hypothetical protein	Hypothetical protein Ct425	*CPN0512*	0512	Q9Z840	5.13/70238	5.11/65008 5.14/64608	
Hypothetical protein	Hypothetical protein Ct429	*CPN0518*	0518	Q9Z834	5.49/38311	5.59/38750	
Hypothetical protein	Hypothetical protein Ct398	*CPN0525*	0525	Q9Z827	8.57/29590	8.39/26972 *	
Hypothetical protein	Hypothetical protein Ct456	*CPN0572*	0572	Q9Z7Y1	5.71/77579	5.68/99200 5.72/98558	
Hypothetical protein	Hypothetical protein Ct504	*CPN0623*	0623	Q9Z7T1	4.90/31059	4.85/39913	
Hypothetical protein	Hypothetical protein Ct538	*CPN0658*	0658	Q9Z7P6	5.28/26288	5.10/25404	
Hypothetical protein	Hypothetical protein	*CPN0677*	0677	Q9Z7M7	7.16/40590	7.28/40508	
Hypothetical protein	Hypothetical protein Ct691	*CPN0681*	0681	Q9Z7M3	5.14/25887	5.15/22279	
Hypothetical protein	Hypothetical protein Ct671	*CPN0705*	0705	Q9Z7K0	4.61/30951	4.64/38835	
Hypothetical protein	Hypothetical protein Ct666	*CPN0710*	0710	Q9Z7J5	5.10/9347	4.67/9435	
Hypothetical protein	Hypothetical protein Ct665	*CPN0711*	0711	Q9Z7J4	6.27/9422	6.45/9565 *	
Hypothetical protein	Hypothetical protein Ct633	*CPN0713*	0713	Q9Z7J2	4.52/14173	4.28/11129 4.41/11404	
Hypothetical protein	Hypothetical protein Ct659	*CPN0720*	0720	Q9Z7I5	9.40/8893	9.61/9637	
Hypothetical protein	Hypothetical protein Ct622	*CPN0728*	0728	Q9Z7H7	4.85/68218	4.73/64907 4.76/64807	
Hypothetical protein	Hypothetical protein Ct635	*CPN0742*	0742	Q9Z7G3	5.56/16478	5.59/13682	
Hypothetical protein	Hypothetical protein Ct632	*CPN0746*	0746	Q9Z7F9	5.80/61146	5.70/58763 5.72/58686	
Hypothetical protein	Hypothetical protein – C.tr	*CPN0797*	0797	Q9Z7A9	5.56/38425	5.15/36848	P
Hypothetical protein	Hypothetical protein Ct579	*CPN0803*	0803	Q9Z7A3	5.57/21270	5.36/18926 5.59/19085 5.74/19149	
Hypothetical protein	Hypothetical protein Ct579	*CPN0808*	0808	Q9Z798	9.38/44477	9.27/43565 * 9.38/43438 *	
Hypothetical protein	Hypothetical protein Ct734	*CPN0875*	0875	Q9Z731	6.01/24486	4.36/11108	L
Hypothetical protein	Hypothetical protein Ct768	*CPN0912*	0912	Q9Z6Z4	6.08/63073	6.11/58457	
Hypothetical protein	Hypothetical protein Ct768	*CPN0939*	0939	Q9Z6W7	4.59/17570	4.52/15851	
Hypothetical protein	Hypothetical protein Ct373	*CPN1032*	1032	Q9Z6M7	5.61/21655	5.78/13030 6.15/12933	
Hypothetical protein	Hypothetical protein	*CPN1040*	1040	Q9Z6L9	5.77/25766	5.60/22306 5.64/21369	
Hypothetical protein	Hypothetical protein	*CPN1054*	1054	Q9Z6K5	5.37/93456	5.73/88250	
Hypothetical protein	Hypothetical protein	*CPN1058*	1058	Q9Z6K1	6.29/41332	5.93/39392	P

Table 3. Continued

Category according to the CGP	Protein name (SW/Tr)	Gene	Nb[a]	Acc.[b]	Theor. p*I*/M_r[c]	Obs. p*I*/M_r[d]	P/L[e]
Other categories:							
Miscellaneous enzymes/conserved prot.	YbaB family hypothetical protein	*ybaB*	0039	Q9Z9E2	4.99/10820	5.02/10189	
Miscellaneous enzymes/conserved prot.	ATPase	*phoH*	0106	Q9Z975	7.74/48349	7.89/41341 *	
Miscellaneous enzymes/conserved prot.	Metal-dependent hydrolase	*phnP*	0479	Q9Z872	5.40/30230	5.32/30614	
Miscellaneous enzymes/conserved prot.	HIT family hydrolase	*ycfF*	0488	Q9Z863	5.46/12071	5.18/10189	
Miscellaneous enzymes/conserved prot.	Probable phosphatase/kinase	*yacE*	0611	Q9Z7U3	5.28/22913	5.21/21606	
Miscellaneous enzymes/conserved prot.	Hydrolase/phosphatase homolog	*CPN0917*	0917	Q9Z6Y9	5.13/17410	5.10/18894	
Miscellaneous enzymes/conserved prot.	Probable D-amino acid dehydrogenase	*CPN1030*	1030	Q9Z6M9	5.51/38098	5.63/42890	
HAD superfamily	HAD superfamily hydrolase/phosphatase	*CPN0407*	0407	Q9Z8D6	5.30/33602	5.23/32224	
Protein folding, assembly, modification:							
Chaperones	DnaJ	*dnaJ*	0032	Q9Z9E9	7.04/42109	6.91/41102 * 7.20/40942 *	
Chaperones	GroEL	*groEL*	0134	P31681	5.29/58204	5.13/56606 5.16/56954 5.20/56806 5.24/56732 5.29/56806 5.34/55274	
Chaperones	GroES	*groES*	0135	P31682	4.64/11316	4.64/12042	
Chaperones	GrpE	*grpE*	0502	Q9Z849	4.88/21291	4.79/22471	
Chaperones	DnaK	*dnaK*	0503	P27542	4.99/71354	4.85/67043 4.88/67146 4.91/66837 4.95/66528 4.99/66631	
Chaperones	DksA	*dksA*	0534	Q9Z818	4.97/13976	4.82/13030	
Proteases	Clp proteinase ATPase (clpB)	*clpB*	0144	Q9Z939	5.41/96928	5.38/91802 5.42/91143	
Proteases	Leucyine aminopeptidase A (LAP)	*pepA*	0385	Q9Z8F8	5.95/54510	5.78/49903 6.04/49518	
Proteases	ClpC protease	*clpC*	0437	Q9Z8A6	6.17/95009	6.45/89691 *	
Proteases	Endopeptidase Clp1	*clpP_1*	0520	Q9Z832	5.58/21020	5.57/20749 5.74/20724	
Proteases	Dihydrolipoamide succinyl-transferase	*sucB_2*	0527	Q9Z825	5.18/42486	5.14/47594 5.20/47594	
Proteases	Aminopeptidase P	*pepP*	0813	Q9Z793	5.88/39992	5.73/35932	
Proteases	Endopeptidase Clp2	*clpP_2*	0847	Q9Z759	5.23/22080	4.99/21134 5.14/21005	
Proteases	DO serine proteinase	*htrA*	0979	Q9Z6T0	7.04/52311	6.57/47818	P
Proteases	ATP-dependent zinc proteinase (C-terminal)	*ftsH*	0998	Q9Z6R1	5.90/101771	5.31/40695	P

Table 3. Continued

Category according to the CGP	Protein name (SW/Tr)	Gene	Nb[a)]	Acc.[b)]	Theor. pI/M_r[c)]	Obs. pI/M_r[d)]	P/L[e)]
Protein isomerases	Thioredoxin disulfide isomerase	*CPN0926*	0926	Q9Z6Y0	8.47/18903	7.02/11325 * 8.57/13055 *	L
Protein isomerases	Predicted disulfide bond isomerase	*CPN0933*	0933	Q9Z6X3	6.48/40493	6.29/34272 *	P
Transcription:							
RNA degradation	RNAse III	*rnc*	0054	Q9Z9C7	5.31/26088	5.27/23833	
RNA degradation	Polyribonucleotide nucleotidyltransferase	*pnp*	0999	Q9Z6R0	5.38/75347	5.29/66119 5.34/65916	
RNA elongation, termination factors	Transcription anti-termination	*nusG*	0076	Q9Z9A5	5.06/20822	5.06/18831	
RNA elongation, termination factors	*N*-utilization protein A	*nusA*	0316	Q9Z8M2	5.23/49037	5.23/48092 5.32/43833	
RNA elongation, termination factors	Transcription termination factor	*rho*	0610	Q9Z7U4	6.80/51819	6.82/50000 * 7.10/49903 *	
RNA polym., transcript. regulator	Sigma regulatory factor	*rsbV_1*	0511	Q9Z841	5.57/12764	5.58/11490	
RNA polym., transcript. regulator	RNA plymerase alpha-subunit	*rpoA*	0626	Q9Z7S8	5.07/41862	5.05/41139 5.10/40991	
RNA polym., transcript. regulator	Sigma regulatory factor – histidine kinase	*rsbW*	0670	Q9Z7N4	5.46/15991	5.49/13104	?
RNA polym., transcript. regulators	HTH transcription reg. prot. and reciever dom.	*tctD*	0750	Q9Z7F6	5.28/26288	5.27/25750	
Translation:							
Amino acyl tRNA synthesis	Glu-ADT subunit C	*gatC*	0002	Q9Z9G8	4.29/11353	4.29/10056	
Amino acyl tRNA synthesis	Glu-ADT subunit A	*gatA*	0003	Q9Z9G7	6.35/53596	6.57/47818 *	
Amino acyl tRNA synthesis	Glu-ADT subunit B	*gatB*	0004	Q9Z9G6	5.19/54599	5.07/48379 5.11/48285 5.16/48285	
Amino acyl tRNA synthesis	Methionine-tRNA ligase (METRS)	*metG*	0122	Q9Z959	5.18/63367	5.14/51655	
Amino acyl tRNA synthesis	Aspartate-tRNA ligase (ASPRS)	*aspS*	0662	Q9Z7P2	5.44/66345	5.37/61783 5.38/60651	
Amino acyl tRNA synthesis	Threonine-tRNA ligase (THRRS)	*thrS*	0806	Q9Z7A0	5.60/72685	5.57/65814 5.63/65410	
Amino acyl tRNA synthesis	Serine-tRNA ligase (SERRS)	*serS*	0870	Q9Z736	5.59/48231	5.59/44312	
Amino acyl tRNA synthesis	Cysteine-tRNA ligase (CYSRS)	*cysS*	0932	Q9Z6X4	5.57/53929	5.57/50854 5.61/50721	
Peptide chain init., elong., term.	EF-Tu	*tufA*	0074	Q9Z9A7	5.43/43005	5.05/37622 5.12/36767 5.12/37581 5.15/41740 5.26/43046 5.39/46057 5.47/45580 5.58/45398	
Peptide chain init., elong., term.	Peptide chain release factor 1 (RF-1)	*prfA*	0113	Q9Z968	5.63/40150	5.69/42158	

Table 3. Continued

Category according to the CGP	Protein name (SW/Tr)	Gene	Nb[a)]	Acc.[b)]	Theor. p*I*/M_r[c)]	Obs. p*I*/M_r[d)]	P/L[e)]
Peptide chain init., elong., term.	Elongation factor P 1 (EF-P1)	*efp_1*	0184	Q9Z900	4.68/20673	4.68/20800	
Peptide chain init., elong., term.	Elongation factor G (EF-G)	*fusA*	0550	Q9Z802	5.23/76588	5.22/76093 5.25/76659	
Peptide chain init., elong., term.	Elongation factor TS (EF-Ts)	*tsf*	0697	Q9Z7K8	5.30/30393	5.11/30330 5.20/30414	
Peptide chain init., elong., term.	Ribosome releasing factor (RRF)	*rrf*	0699	Q9Z7K6	8.44/20169	8.37/20293 *	
Peptide chain init., elong., term.	Polypeptide deformylase (PDF)	*def*	1067	Q9Z6J2	5.79/21017	5.70/22471 5.79/22197	
Peptide chain init., elong., term.	Elongation factor P (EF-P2)	*efp_2*	0895	Q9Z711	4.85/21478	4.85/22581	
Ribosomal proteins	50S ribosomal protein L10	*rl10*	0079	Q9Z9A2	6.73/18429	7.02/16497 *	
Ribosomal proteins	50S ribosomal protein L7/L12	*rl7*	0080	Q9Z9A1	5.02/13461	4.89/15101	
Ribosomal proteins	30S ribosomal protein S1	*rs1*	0315	Q9Z8M3	5.16/65020	5.15/65916 5.18/65814	
Ribosomal proteins	30S ribosomal protein S13	*rs13*	0628	Q9Z7S6	10.75/13965	7.35/10501 *	
Ribosomal proteins	50S ribosomal protein L9	*rl9*	0953	Q9Z6V3	9.00/18723	8.67/16331	

a) CGP gene number (Cpn*xxxx*)
b) Accession numbers are from the SWISS-PROT or TrEMBL database
c) Theoretical p*I* and M_r were calculated using the Compute pI/Mw Tool at the ExPASy server (predicted leader peptides were not cleaved off before calculation).
d) Oberserved p*I* and M_r Data are from the IPG 4–7 except for those marked × (IPG 6–11) and # (NL IPG 3–10). Data are listed for all identified isoforms.
e) Proteins marked P were predicted by the Signal P server to be transported over the inner membrane. Proteins marked L contain the ProSite pattern for lipid modification.

served phylogenetically and eukaryotic homologous are named HSP(M_r) (heat shock protein of *xx* kDa). In *E. coli*, the chaperones GroEL (HSP60) and GroES function in a complex which catalyze folding of proteins under hydrolysis of ATP (for review see Martin [19]). The *C. pneumoniae* GroEL and GroES each make up more than 1% of the protein visualized in the NL IPG 3–10 image (Fig. 2; GroEL). The genes for GroES and GroEL are located in the *groE* operon (contig 2.6, genes Cpn0135, Cpn0134), however, two homologs of the *groEL* gene are present in other regions of the genome (contig 9.7 gene Cpn0777 p*I*/M_r 5.27/56.6) and 11.2 gene Cpn0898 pI/M_r 5.89/56.9). The amino acid sequence encoded by these genes have only 36% and 27% identity to the GroEL from the *groE* operon. No fragments of the GroEL homologs were identified.

The chaperones DnaK (HSP70) and DnaJ bind to newly synthesized peptide chains cotranslationally to prevent misfolding and aggregation. Under hydrolysis of ATP, the high affinity complex of DnaK/DnaJ/ADP/protein is dissociated by GrpE. The protein is liberated either folded or to the GroEL/ES complex for folding. *C. pneumoniae* DnaK makes up 1.8% of the proteins visualized in the image. Several isoforms of DnaK are seen (Fig. 2; DnaK). It is known that *E. coli* DnaK can autophosphorylate at threonine-199 and thereby increase its substrate binding affinity [20]. This can also be the case with *C. pneumoniae* DnaK, but it does not account for the high number of isoforms observed. Several isoforms have also been observed of *C. trachomatis* DnaK [21]. The chaperones DnaJ and GrpE were also identified. The protein DksA was found in low amounts. DksA has the confusing name "DnaK suppressor protein", because high expression of DksA can suppress the filamentation growth of an *E. coli* DnaK deletion mutant. However, DksA can not restore all DnaK functions, for instance, growth of λ-phage, but high expression of DksA can restore temperature sensitive growth of DnaJ and GrpE

mutants [22]. The Clp protein complex is important for protein degradation. ClpP is the proteolytic component which in the presence of ATP together with ClpA or ClpX can form a eukaryotic proteasome-like structure [23, 24]. ClpPA, ClpPB, ClpX are chaperone-like ATPases that provide substrate specificity to the complex. Two *clpP* homologs are present in the *C. pneumoniae* genome called *clpP_1* and *clpP_2* and both are present in EB. Of Clp ATPases, *clpB, clpC* and *clpX* homologs are present in the genome. Both ClpB and ClpC were detected.

3.4.2 Cell envelope and Pmps

Several envelope proteins have been identified. MOMP, Omp2, Omp3, Pmp10 and Pmp11 are all known to be expressed from earlier studies [25–27]. Except Omp3, all of these are found in the gel. The Pmps belong to a superfamily of proteins consisting of nine members in *C. trachomatis* and 21 members in *C. pneumoniae* [10]. Considering the small size of the genome it is remarkable that such a large number of related proteins is maintained and that the family is expanded in *C. pneumoniae*. By RNA experiments, Stephens and Lindquist [28] have shown transcription of all nine Pmps in *C. trachomatis*. Here, translation has been shown for ten *C. pneumonia* Pmps (Pmp2, 6, 7, 8, 10, 11, 13, 14, 20 and 21). MOMP, Omp2 and the identified Pmps are all present in high amounts in the gels and for all of these proteins a train of spots is observed rather than a single spot (Fig. 2; Pmp8, Pmp10). Whether this appearance is due to the presence of several isoforms in the bacteria or to some artefactual phenomenon is not known. Care has been taken not to heat samples in order to avoid carbamylation and the trains are observed in all gels that have been made. If many isoforms really are present in EB it is interesting why these membrane proteins are particularly prone to modification.

Other members of this category are proteins involved in synthesis of lipopolysaccharide and fatty acid. Both these pathways are well represented in EB. Only one gene product involved in peptidoglycan biosynthesis has been identified. This is the product from the fused gene of *murC* and *ddlA*. Muramic acid has only been detected in *Chlamydia* in very small amounts [29], but penicillin binding proteins have been identified in earlier studies [30].

3.4.3 Cellular processes

Interaction between bacterial pathogens and their host is mediated by membrane-associated proteins or proteins secreted into the host cell. For Gram-negative bacteria four pathways of protein secretion have been described: type I, II, III and IV. The type I secretion apparatus spans the periplasmic space and transports proteins carrying a *C*-terminal signal sequence to the extracellular space. No processing of the secreted proteins is involved. Type II secretion is a two-step process in which the proteins for secretion are first transported over the inner membrane by the *sec* system. In the periplasmic space the *N*-terminal leader sequence necessary for transport over the inner membrane is cleaved off by a signal peptidase. Transport over the outer membrane requires a set of transport proteins different from the *sec* apparatus. Type II secretion is the primary transport mechanism for degradative enzymes. Type III secretion, which is specific for pathogenic bacteria, does not involve processing of the secreted proteins and the type III apparatus like the type I apparatus spans the periplasmic space. The type III apparatus has additionally been suggested to involve a structure on the surface of the bacteria which could serve as a pipeline for translocation of proteins. For *P. syringae* a pilus-like structure is formed on the surface when the bacteria are grown on a type III inducing media. These structures are not observed for type III secretion mutant strains [31]. Such a mechanism would be especially important for *Chlamydia* spp. since they are obligate intracellular and proliferate in a vacuole in the host cell. Type III pipelines can be their way of transporting effector proteins across the eukaryotic membrane. Electron microscopy observations of pili-like protrusions from the outer membrane of *C. psittaci* EB and RB done by Matsumoto [32] and on *C. pneumoniae* by Miyashita *et al.* [33] have been interpreted as projections of the type III secretion apparatus [34]. For a comprehensive review on bacterial type III secretion see Huek [35]. Type IV secretion resembles type II by *sec*-dependent transport over the inner membrane and cleavage of a leader sequence in the periplasmic space. Transport across the outer membrane, however, is facilitated by the secreted protein itself, hence these proteins are called autotransporters. The *C*-terminal of the protein forms a pore in the membrane through which the *N*-terminal is transported and subsequently possibly released after an autoproteolytic cleavage. For review of autotransport see Henderson *et al.* [36].

From the type I secretion apparatus three ABC transporter proteins were identified. From the type II secretion apparatus three proteins were identified including GspD which is a homolog of the membrane pore protein PulD from *Klebsiella oxytoga*. From the type III secretion apparatus four members were identified: (i) YscC which is a homolog of the *Yersinia* outer membrane pore protein (unified nomenclature is SctC [35]); (ii) YscN which is an ATPase homolog thought to be connected to the inner

membrane and involved in the delivery of energy for secretion (SctN [35]); (iii) YscL which is homolog to a protein probably located in the periplasmic space of *Yersinia* (SctL [35]); (iv) low calcium response E which is a substrate of the secretion pathway thought to be associated with the outer membrane and may possess regulatory function.

C. pneumoniae may also have type IV secretion. We speculate that some of the Pmps, which are predicted to be transported over the inner membrane by the *sec* system and have a *C*-terminal outer membrane anchor, can be type IV autotransporters. They are predicted to form a *C*-terminal beta-barrel and have a high GNAST percentage which is common for autotransporters. Furthermore, they contain a 4–23 times repeated motif of GGA[ILV], which is repeated more than three times in only two known non-chlamydial bacterial proteins [37]: (i) rOmpA, involved in binding of *Rickettsia conorii* to host cells; (ii) YFAL from *E. coli* the function of which is unknown but which contains a *C*-terminal domain homologous to *E. coli* AIDA-1. AIDA-1 cannot be aligned to the Pmps, but like for the Pmps the majority of tryptophan residues are found in the *C*-terminal, and the *N*-terminal contains a region of repeated glycine-rich motifs. AIDA-1 and rOmpA autotransporters [38]. Beside transport proteins, the two detoxification proteins, superoxide dismutase and TSA peroxidase and three proteins involved in cell division have been identified.

3.4.4 Energy metabolism

For many years *Chlamydia* has been thought to rely entirely on energy delivered by the host cell. The CGP revealed genes for generating ATP and reducing power as well as for glycolysis, glyconeogenesis and in part tricarboxylic acid cycle. Following the uptake of EB into the host cell energy is required to promote the differentiation into RB. Type III secretion from EB would also require energy. EB have been shown to carry a pool of ATP [39] and this could be supplemented by ATP generated from stored glycogen until uptake from the host cell can be established by RB as suggested by Iliffe-Lee and McClarty [40]. EB are not capable of obtaining ATP from the host cell [41].

We have identified several key enzymes of energy metabolism in EB including transketolase and glucose-6-phosphate dehydrogenase from the pentose phosphate pathway and ATP synthase B. From glycolysis and glyconeogenesis, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase were found together with four other proteins. In addition, five proteins from the tricarboxylic acid cycle and five proteins from the pyruvate dehydrogenase pathway including pyruvate dehydrogenase alpha were identified.

3.4.5 Amino acid biosynthesis and nucleotide metabolism

The pathways of amino acid biosynthesis are incomplete in *Chlamydia* and thus proposed to carry out other functions than *de novo* biosynthesis of amino acids [9]. No proteins involved in amino acid biosynthesis were found. Three monophosphate kinases were found as well as the nonspecific nucleoside diphosphate kinase *(ndk)*. These proteins allow for the reuse of (d)NMPs and (d)NDPs. Thioredoxin and thioredoxin reductase are other examples of identifications from this category.

3.4.6 Cell intermediary metabolism

Only three members of this category were identified. These were: glycogen phosphorylase which is important for carbohydrate metabolism, phosphoenolpyruvate carboxykinase which is a rate limiting enzyme in gluconeogenesis, and inorganic pyrophosphatase which catalyzes the formation of orthophosphate from pyrophosphate.

3.4.7 DNA replication, modification, repair and recombination

Only a total of five proteins or 9% of the members of this category were found, RecA and four proteins involved in replication: the DNA polymerase III beta-chain, a gyrase, a helicase and a single-strand binding protein. No histone-like proteins were found although they should be present in high amounts [8]. This can be due to their basic character (p*I*/M_r is 10.7/13 kDa for HctA and 12.4/19 kDa for HctB). An unidentified spot of high volume is present in the area of the predicted localization of HctA but several ribosomal proteins are also expected in this area and besides HctA contains only two methionines including the start methionine and no cysteines. HctB is not expected to be inside the boundaries of the investigated pH range. That no more than five proteins are found in the DNA category can be due to a nonreplicating state of the EB or low intra-cellular abundance.

3.4.8 Transcription

In the transcription category 21% of the members have been identified. These proteins include RNA polymerase alpha-chain in high amounts (1.3%) and two sigma regulatory factors: RsbV1 and RsbW, both barely detectable. The genome of *C. pneumoniae* contains three predicted

sigma factors, RpoD (σ^{66}) and two alternative factors, RpsD (σ^{28}) and RpoN (σ^{54}). The sigma factors have been speculated to be involved in regulation of developmental stage specific transcription. Four genes have been predicted to encode sigma regulating factors, *rsbVI, rsbV2, rsbw* and *rsbU*.

RT-PCR analysis of *C. trachomatis* L2 sigma factors and regulators by Douglas and Hatch [42] showed that transcripts of *rpoC* (RNA polymerase beta-chain), and *rpoD* were present at all times post infection and that *rpoD* transcripts decreased very late in the developmental cycle. Surprisingly, transcripts were also detected in EB but only in very small amounts for *rpoC* and *rpoD*. Based on these findings they suggested that a decrease in RpoD protein levels late in the developmental cycle contributes to the inactivity of EB. It is implicated that high expression of *rpoD* is required upon infection. We find RpoA in high amounts in EB and hence speculate that RpoC despite the low level of transcripts could be present too, and remind that absence of RNA does not imply absence of protein. With the exception of *rsbV2*, transcripts of the alternative sigma factors and putative regulatory factors were only detected by Douglas and Hatch during the exponential growth phase of RB. These findings indicate that the regulatory effect of these proteins is not stage-specific.

Mathews *et al.* [43] have used lightcycler technology to quantify the RNA levels of sigma factors and regulators in *C. trachomatis* L2. They also found a constitutive expression of *rpoD* but found that expression of *rpsD* peaked at 12 hpi whereas expression of *rpoN* peaked at 30 hpi. Based on these results they suggested that RpoN is involved in regulation of RB to EB conversion. Identification of sigma factors and sigma regulatory factors and a direct measure of protein levels throughout the developmental cycle would lead to further insight in their role in regulation of expression in *Chlamydia*.

3.4.9 Translation

Twenty-four percent of the proteins in the translation category were identified including essential components such as elongations factors Tu, Ts, P and G. EF-Tu accounts for 5% of the proteins visualized in the NL IPG 3–10 image. Only two proteins were present in higher amounts, RL7 (7%) and MOMP (20%). Four other ribosomal proteins were found together with the ribosomal release factor and peptide chain release factor. Other proteins found were five tRNA ligases and subunits A, B and C of the Glu-tRNA Gln amidotransferase. All together, a remarkably high number of translational proteins are present in EB.

3.4.10 Hypothetical proteins

Thirty-one hypothetical proteins were found. Four of these (Cpn0104, Cpn0797, Cpn0875 and Cpn1058) contained a signal peptidase I cleavage site and were thus predicted to be transported over the inner membrane. In addition, Cpn0875 was predicted to be lipid-modified. Five were not found to have an ortholog in *C. trachomatis*. Interestingly, many of the hypothetical proteins were highly abundant (Cpn0720, Cpn1032, Cpn0808, Cpn0677) (Table 2). The gene *cpn0808* is located in close proximity of a type III chaperone homolog, *cpn0811* (homologous to *lcrH-1*) and the hydrophobicity profile of Cpn0808 resembles that of YopB and YopD from *Yersinia* which are secreted type III effector proteins. Therefore, Cpn0808 has been suggested to be secreted by the type III secretion system [44]. The presence of high amounts of Cpn0808 in purified EB implies that it has been retained in the bacteria during the late phase of the developmental cycle. If Cpn0808 is a type III effector protein, it may reside in EB waiting for the bacteria to get in contact with a host cell before secretion is initiated.

3.5 Throughput and general remarks

More than 70% of the analyzed samples pooled from two gels led to the identification of a chlamydial protein. Especially since it is difficult to obtain pure protein samples, due to the obligate intracellular nature of *Chlamydia*, this must be considered a high identification rate and proves the value of purification of EB as well as sample clean-up of peptides for MS analysis. One of the advantages of proteomics is that the examination of protein expression can be addressed in the most direct way, but it cannot be deduced from proteomics whether a protein not found in a gel is not expressed. Proteins cannot be cloned and can be: (i) present in undetectable amounts; (ii) outside the boundaries of the separation range; (iii) lost during the sample preparation procedure, or (iv) the protein in question can reside in a protein spot which is not identified. In other words, proteomics is conclusive on presence but inconclusive on absence of proteins. Thus, presumed lack of expression of specific proteins depicted from proteome analysis should be further investigated by transcription analysis. Furthermore, proteomics is inconclusive on functional competence of proteins and proteins showing interesting expression patterns should be analyzed by functional assays if possible.

3.6 2-D database

The results have been made available on the www showing images of all investigated pH ranges with identified spots highlighted and linked to the SWISS-PROT and

TrEMBL databases as well as a file containing information on identifications. Furthermore, a list of all identified proteins sorted into categories has been made available containing information on protein and gene names, CGP gene number, SWISS-PROT/TrEMBL accession number, Signal-P prediction of transport across the inner membrane, prediction of lipid modification by ProSite search, theoretical p*I*/M_r before and after cleavage, observed p*I*/M_r and content of Cys/Met. The address of the web site is: www.gram.au.dk.

4 Concluding remarks

Some important findings are (i) the high number of energy metabolizing proteins and nucleotide metabolizing proteins; (ii) the remarkably high number and content of proteins involved in transcription and translation; (iii) many predicted outer membrane proteins including eight Pmps; (iv) evidence for expression of many hypothetical proteins; (v) key proteins of the type III secretion apparatus.

By these findings, we have added value to the theory that EB are capable of metabolizing energy and, thereby, provide fuel for protein expression and active transport of proteins at the very beginning of the developmental cycle. We furthermore suggest that EB carry a functional transcription and translation apparatus ready to be activated at the time of infection. EB are in most publications described as being metabolically inactive. The presence of a broad range of proteins involved in various pathways, and the fact that Douglas and Hatch [42] have found transcripts in EB, may lead to the question whether EB really are "metabolically inactive" dormant-like bodies. We suggest this term is moderated to "non-dividing".

This study was initiated to provide a reference map for further investigations. As we have used commercially available IPGs, the generated web site hopefully can be of use for interlaboratory comparison [45]. For an obligate intracellular bacteria as *Chlamydia* for which the dividing form (RB) is difficult to isolate it is a great advantage that protein levels can be monitored in a direct way. Proteins can by pulse-chase labeling be followed in time, with respect to synthesis and with respect to subsequent processing. Besides, changes in protein profiles implied by amino acid depletion, stress or different growth conditions such as cultivation in different cell lines can be monitored.

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The expression, processing and localization of polymorphic membrane proteins in *Chlamydia pneumoniae* strain CWL029

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Abstract

Background: Chlamydiae are obligate intracellular bacteria, which are important human pathogens. Genome sequences of *C. trachomatis* and *C. pneumoniae* have revealed the presence of a *Chlamydia* specific gene family encoding polymorphic outer membrane proteins, Pmps. In *C. pneumoniae* the family comprises twenty-one members, which are all transcribed. In the present study, the expression, processing and localisation of the sixteen full-length Pmps in *C. pneumoniae* strain CWL029 have been further investigated by two-dimensional gel electrophoresis and immunofluorescence microscopy.

Results: Ten Pmps were identified in elementary bodies (EBs). Eight of these were investigated with respect to time dependent expression and all were found to be up-regulated between 36 and 48 hours post infection. Antibodies against Pmp6, 8, 10, 11 and 21 reacted with chlamydiae when infected cells were formalin fixed. Pmp6, Pmp20 and Pmp21 were found in cleaved forms, and the cleavage sites of Pmp6 and Pmp21 were identified.

Conclusions: The Pmps are heavily up-regulated at the time of conversion of RB to EB, and at least ten Pmps are present in EBs. Due to their reaction in formalin fixation it is likely that Pmp6, 8, 10, 11 and 21 are surface exposed. The identified cleavage sites of Pmp6 and Pmp21 are in agreement with the theory that the Pmps are autotransporters.

Background

Chlamydiae are pathogenic gram-negative bacteria of which *C. pneumoniae* causes upper and lower respiratory tract infections in humans [1]. Going through a developmental cycle the chlamydiae alternate between infective elementary bodies (EBs) and replicative reticulate bodies (RBs) [2]. The bacteria are obligate and intracellular, residing inside a specialized phagosome, named the chlamydial inclusion. The duration of the developmental

cycle for *C. pneumoniae* cultivated in cell culture is about 72 hours [3].

The *C. pneumoniae* CWL029 genome sequence revealed the presence of a gene family, the *pmp* family, consisting of 21 members [4] that were paralogous to the *pmps* found in *C. trachomatis* [5] and *C. psittaci* [6–9]. The *C. psittaci* Pmps have been analysed by two-dimensional electrophoresis in an earlier study [37]. The Pmps are two-domain proteins with similarity to autotransporter proteins [4,10,11]. They are characterized by a high frequency of the two sequences FxxN and GGAI in the N-terminal part (twelve and seven repeats on average, respectively) [4], and their C-terminal part shows the characteristics of a β-barrel [10]. The GGAI motif and the prediction of a C-terminal β-barrel suggest that the Pmps are autotransporters, transporting an N-terminal passenger domain through a pore formed by their C-terminal part [10,12]. Seventeen *C. pneumoniae* Pmps contain a signal peptidase I cleavage site directing transport over the inner membrane, and two Pmps contain a signal peptidase II cleavage site suggesting lipid modification [4].

The Mw of most Pmps in *C. pneumoniae* strain CWL029 is predicted to be just below 100 kDa, but three are larger: Pmp6 is 142 kDa, Pmp20 is 178 kDa and Pmp21 is 167 kDa and Pmp12 is only 56 kDa. Four genes (*pmp3, 4, 5* and *17*) contain a mutation resulting in premature stop. In *C. pneumoniae* strain AR39 [13] and J138 [14]*pmp6* contains 393 less base pairs, and in strain J138 Pmp2 and Pmp4 contain frame shift mutations [14].

Variation of membrane protein expression is thought to provide protection against the immune system of the host [15] and it has been suggested that the Pmps may provide such a protection of chlamydiae. This was indicated by the findings of Birkelund *et al.* [16] and Pedersen *et al.* [17] who observed differential expression of Pmp10.

Transcripts have been detected from all Pmp genes in *C. trachomatis* [18] as well as *C. pneumoniae* [19]. As part of a large-scale proteome analysis [20], ten Pmps (Pmp2, 6, 7, 8, 10, 11, 13, 14, 20 and 21) or fragments of these were identified by mass spectrometry (MS) in *C. pneumoniae* CWL029 EBs. The positions of these in the 2-D protein profile can be viewed at [http://www.gram.au.dk]. In addition to these, Montigiani *et al.* [21] identified Pmp16 by MS. Grimwood *et al.* [19] found Pmp2, 6, 7, 8, 9, 11, 13, 14, 15, 16, and 18 to be present in *C. pneumoniae* CWL029 EBs by immunoblotting (IMB) using antibodies raised against synthetic 20-mer peptides. Pmp1, 19, 20 and 21 were not detected although full-length genes encode these. Pmp6 migrated around 100 kDa although it theoretically should be 144 kDa.

The detection of Pmp6 forty kDa below the predicted value has been interpreted by Henderson and Lam [10] as an indication that an N-terminal passenger domain is cleaved off, which is frequently seen for autotransporters. In strains AR39 and TW183, sharing the 393 base pair deletion, Pmp6 was detected at the resulting theoretical full-length position of 130 kDa by Grimwood *et al.* [19].

In the study described in this paper, 2-D PAGE was used to elucidate the expression pattern of the *C. pneumoniae* Pmps in CWL029 at different times during the developmental cycle. Pmp6, Pmp20 and Pmp21 were found to be cleaved and the cleavage sites of Pmp6 and Pmp21 were identified. IMF was used to determine whether Pmps could be detected at the surface of the bacteria.

Results

The proteome study by Vandahl *et al.* [20] was not conclusive on the absence of any Pmps. Hence, we performed 2-D immnoblotting (IMB) to identify the 2-D PAGE position of expressed Pmps not earlier mapped. Pmp3, 4, 5, 12 and 17 were omitted from the study because these are truncated. Polyclonal antibodies (pAbs) were obtained by immunization of rabbits with recombinant proteins produced in *E. coli* (table 1). Antigens were raised against the N-terminal part of Pmp6, 7, 8, 10, 13, 14, 15, 16, 18, 19, 20 and 21 because antibodies raised against full length Pmps showed cross-reactivity. The N-terminal part is the most variable and has been detected at the surface for *C. psittaci* Pmps [9]. All the obtained pAbs reacted in 1-D IMB with the recombinant Pmps to which they were raised (data not shown). The antigen for IMB was EB lysate. Reacting spots (figure 1A) were identified by comparison to a reference gel upon autoradiography of the blot (figure 1B) [22] or by MS analysis of spots excised from corresponding gels.

The proteins detected by each antibody are listed in table 1 using bold for strong reaction and plain for weaker reaction. PAb203-rPmp10 was mono-specific for Pmp10. The antibodies against rPmp2, rPmp7, rPmp8 and rPmp11 recognized these Pmps (respectively), and additional Pmps as listed in table 1. PAb221-rPmp6 recognized the 91 kDa spot that was earlier identified as originating from Pmp6 [20] and in addition another Pmp6 spot at 60 kDa marked X in figure 1A. PAb236-rPmp20 recognized a spot located just above the 90 kDa Pmps, which was earlier identified as originating from Pmp20 [20]. PAb237-rPmp21 recognized the 91 kDa Pmp6 spot, Pmp13 and a 48 kDa Pmp21 fragment marked Y in figure 1C. The results with antibodies against rPmp1 and rPmp9 were inconclusive and MS identification of these Pmps could not be obtained. Antibodies against rPmp16 and rPmp18 both reacted with Pmp8 and Pmp10 but no other proteins. Antibodies against rPmp15 and rPmp19 did not re-

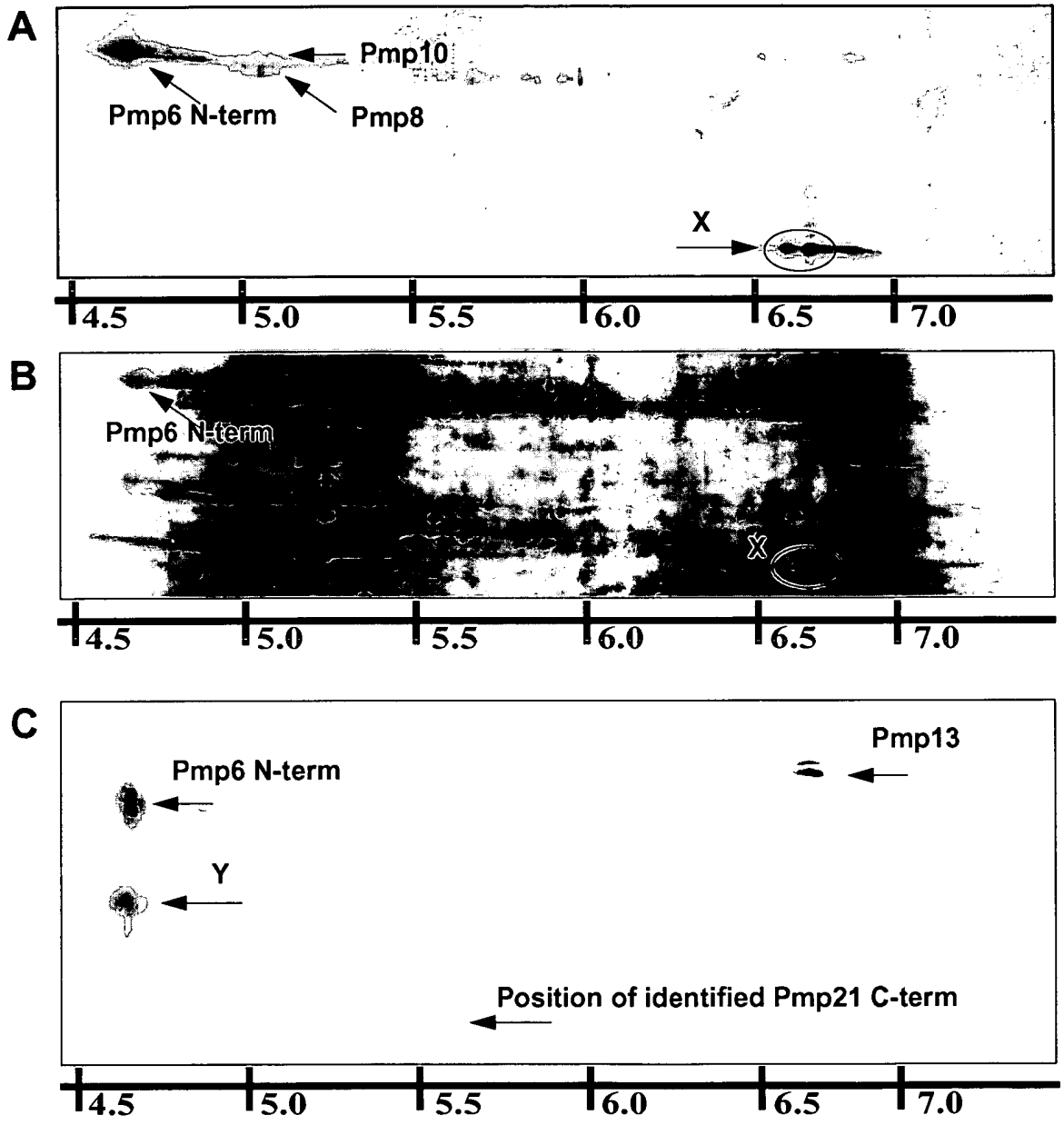

Figure 1
2-D immunoblotting of EB proteins Radiolabeled proteins from EBs purified 72 hpi were separated by 2-D PAGE and electroblotted onto PVDF membranes. The membranes were reacted with polyclonal rabbit antibodies raised against recombinant Pmp. Bound antibodies were visualized by color reagents and membranes were afterwards exposed to X-ray films so that reacting spots could be identified by comparison to an annotated gel. **A:** PVDF blot of EB proteins reacted with Pmp6-pAb221, X shows a C-terminal fragment of Pmp6 recognized by Pmp6-pAb221; **B:** Autoradiography of A; **C:** PVDF blot of EB proteins reacted with pAb237-rPmp21, Y shows an N-terminal fragment of Pmp21 recognized by pAb237-rPmp21.

Table 1: Overview of Pmps, antibodies and immunoblot reactions

pAb	Pmp	Cl.	Fam.	L	aa	cloned	MS	G	M	IMB	MeOH	Form.
204	**1**	I	G	I	923	**26–923**	-	-	I	**1?2 6 7 8 10 13 14 20 21**	X	-
207	**2**	I	G	I	842	**20–842**	X	S	M	**2 6 8 10**	X	-
-	**3***	I	G	I								
-	**4***	I	G	I								
-	**5***	I	G	I								
221	**6**	II	G	I	1408	18–905	N+C	S	M	**6 8 10X**	X	X
220	**7**	II	G	I	937	25–545	X	S		**2 6 7 8 10 11 13**	X	-
201	**8**	II	G	I	931	21–931	X	S	M	**8 10**	X	X
208	**9**	II	G	I	929	**27–929**	-	S	I	**2 6 7 8 9? 10 11 13 14 20**	X	u
203	**10**	II	G	II	929	**20–929**	X	-	M	**10**	X	X
195	**11**	II	G	II	929	**1–929**	X	W	M	**6 11**	X	X
-	**12#**	II	-	I								
222	**13**	II	G	I	974	20–542	X	S	M	*not performed*	X	-
228	**14**	II	H	I	979	25–492	X	S	M	*not performed*	X	-
229	**15**	III	EF	I	939	18–478	-	W	I	-	-	-
230	**16**	III	EF	N	935	1–472	-	S	M	8 10	-	-
-	**17***	III	EF	I								
231	**18**	III	EF	N	893	1–566	-	P		8 10	-	-
234	**19**	IV	A	I	948	22–501	-	-		-	-	-
236	**20**	IV	BC	I	1724	22–1272	X	-	M	20 13	-	-
237	**21**		D	I	1610	52–1129	N+C	-	M	**6 13Y**	X	X

pAb: Antibody number; **Pmp:** Pmp number: *, genes containing a premature stop; #, naturally short gene; shading indicates either * or #. **Cl.**: Cluster in the *C. pneumoniae* genome.**L:** Predicted leader sequences: I, signal peptidase I cleavage site; II, signal peptidase II cleavage site; -, no predicted cleavage site. **Fam.**: Closest related *C. trachomatis* Pmp. **aa:** Amino acids in full-length Pmp. **cloned:** First and last amino acid in the recombinant protein. **MS:** Mass spectrometry identification by: X, peptides dispersed along the entire sequence; N, peptides localized to the N-terminal part; C, peptides localized to the C-terminal part; -, not identified by MS. **G:** Detection in 1D IMB by Grimwood *et al.*: S, strong reaction; W, weak reaction; P, possible reaction; -, no reaction. **M:** Detection by Montigiani *et al.* [24]: M, MS and immunoblot identification; I, immunoblot detection alone. **IMB:** Immunoblotting results: bold numbers indicate strong reaction; X is a C-terminal fragment of Pmp6; Y is an N-terminal fragment of Pmp21; results marked ? were inconclusive. **MeOH:** MeOH fixation IMF at 72 hpi: X, reaction; -, no reaction. **Form.**: Formalin fixation IMF at 72 hpi: X specific reaction; u, unspecific reaction; -, no reaction.

act with any EB proteins. We were thus not able to detect Pmp 15, 16, 18 and 19 by IMB using EB lysates as antigen.

Identification of cleaved Pmps

The antibodies against the high molecular weight Pmps (Pmp6, Pmp20 and Pmp21) recognized protein spots at lower molecular weight than expected. PAb221-rPmp6 recognized a set of spots, X, at 60 kDa (figure 1A) in addition to the earlier identified 91 kDa fragment of Pmp6. MS analysis of a tryptic digest of X revealed peptides from the C-terminal part of Pmp6 (singly underlined peptides, figure 2). In contrast, the mass spectra from the Pmp6 spot at 91 kDa was found to contain peptides solely from the N-terminal part of Pmp6 (doubly underlined peptides, figure 2). To verify the identifications, gels were electroblotted onto PVDF membranes and N-terminal amino acid sequences of the proteins were assessed by Edman degradation. The sequence obtained from X (xVPVVPVAP) confirmed that it was a C terminal fragment of Pmp6 as it matched an internal sequence of Pmp6 (^{872}SVPVVPVAP) (figure 2). The sequence obtained from the N-terminal fragment (xNTDLxSSD) matched a sequence of Pmp6 (^{24}ANTDLSSSD) that confirmed cleavage at the signal peptidase I cleavage site predicted by Signal-P [23,24] (figure 2). As seen in figure 2, these cleavage sites explain the observed pI and Mw coordinates. Also in figure 2, a model of Pmp6 is shown where the internal cleavage site is mapped relative to the predicted domains.

PAb237-rPmp21 reacted with a novel spot pattern, Y, at 66 kDa (figure 1C). A tryptic digest of Y was found to contain solely N-terminal peptides of Pmp21 by MS analysis (amino acids 122–130, 181–202, 203–213, 251–268, 493–510, and 530–548). The earlier identified Pmp21 spot at 48 kDa [20] was found to contain solely C-terminal peptides (amino acids 1205–1223, 1251–1273, 1279–1289, 1295–1311, 1312–1326, 1315–1326, 1400–1417, 1447–1457, 1464–1483, 1465–1483, 1484–1494, 1502–1521, 1504–1521, 1525–1540, 1594–1606). No reaction was observed with the latter spot (figure 1C) explained by the fact that pAb237-rPmp21 was raised against a recombinant protein covering only the N-terminal part of Pmp21, up to amino acid 1129 (table 1). Edman degradation of the 48 kDa spot revealed a sequence matching an internal sequence of Pmp21 (^{1185}SSPTPNKDKA) thereby identifying the cleavage site.

0001 MKYSLPWLLT SSALVFSLHP LMAANTDLSS SDNYENGSSG SAAFTAKETS DASGTTYTLT
0061 SDVSITNVSA ITPADKSCFT NTGGALSFVG ADHSLVLQTI ALTHDGAAIN NTNTALSFSG
0121 FSSLLIDSAP ATGTSGGKGA ICVTNTEGGT ATFTDNASVT LQKNTSEKDG AAVSAYSIDL
0181 AKTTTAALLD QNTSTKNGGA LCSTANTTVQ GNSGTVTFSS NTATDKGGGI YSKEKDSTLD
0241 ANTGVVTFKS NTAKTGGAWS SDDNLALTGN TQVLFQENKT TGSAAQANNP EGCGGAICCY
0301 LATATDKTGL AISQNQEMSF TSNTTTANGG AIYATKCTLD GNTTLTFDQN TATAGCGGAI
0361 YTETEDFSLK GSTGTVTFST NTAKTGGAIY SKGNSSLTGN TNLLFSGNKA TGPSNSSANQ
0421 EGCGGAILAF IDSGSVSDKT GLSIANNQEV SLTSNAATVS GGAIYATKCT LTGNGSLTFD
0481 GNTAGTSGGA IYTETEDFTL TGSTGTVTFS TNTAKTGGAL YSKGNNSLSG NTNLLFSGNK
0541 ATGPSNSSAN QEGCGGAILS FLESASVSTK KGLWIEDNEN VSLSGNTATV SGGAIYATKC
0601 ALHGNTTLTF DGNTAETAGG AIYTETEDFT LTGSTGTVTF STNTAKTAGA LHTKGNTSFT
0661 KNKALVFSGN SATATATTTT DQEGCGGAIL CNISESDIAT KSLTLTENES LSFINNTAKR
0721 SGGGIYAPKC VISGSESINF DGNTAETSGG AIYSKNLSIT ANGPVSFTNN SGGKGGAIYI
0781 ADSGELSLEA IDGDITFSGN RATEGTSTPN SIHLGAGAKI TKLAAAPGHT IYFYDPITME
0841 APASGGTIEE LVINPVVKAI VPPPQPKNGP IASVPVVPVA PANPNTGTIV FSSGKLPSQD
0901 ASIPANTTTI LNQKINLAGG NVVLKEGATL QVYSFTQQPD STVFMDAGTT LETTTTNNTD
0961 GSIDLKNLSV NLDALDGKRM ITIAVNSTSG GLKISGDLKF HNNEGSFYDN PGLKANLNLP
1021 FLDLSSTSGT VNLDDFNPIP SSMAAPDYGY QGSWTLVPKV GAGGKVTLVA EWQALGYTPK
1081 PELRATLVPN SLWNAYVNIH SIQQEIATAM SDAPSHPGIW IGGIGNAFHQ DKQKENAGFR
1141 LISRGYIVGG SMTTPQEYTF AVAFSQLFGK SKDYVVSDIK SQVYAGSLCA QSSYVIPLHS
1201 SLRRHVLSKV LPELPGETPL VLHGQVSYGR NHHNMTTKLA NNTQGKSDWD SHSFAVEVGG
1261 SLPVDLNYRY LTSYSPYVKL QVVSVNQKGF QEVAADPRIF DASHLVNVSI PMGLTFKHES
1321 AKPPSALLLT LGYAVDAYRD HPHCLTSLTN GTSWSTFATN LSRQAFFAEA SGHLKLLHGL
1381 DCPASGSCEL RSSSRSYNAN CGTRYSF

Theoretical values			Observed values		
Fragment	**kDa**	**pI**	**Fragment**	**kDa**	**pI**
Full length	**142.3**	**5.28**	**Full length**	*not observed*	
N-terminal	**84.8**	**4.64**	**Acidic spot**	**91.3**	**4.63**
C-terminal	**57.5**	**6.75**	**Basic spot**	**60.0**	**6.67**

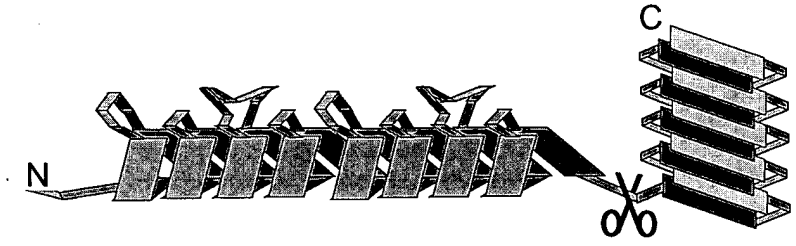

Figure 2
Pmp6 fragments Peptides identified from the acidic protein spot by MS are doubly underlined. Peptides identified from the basic spot are singly underlined. Amino acids from N-terminal sequences obtained by Edman degradation of the fragments are shown in bold. GGAI/L repeats are boxed. Notice that all repeats are located N-terminally of the cleavage site. Theoretical pI and Mw values of fragments were calculated by the Compute pI/Mw tool [http://www.expasy.ch/tools/pi_tool.html]. A schematic drawing of the predicted domains is shown at the bottom, the pair of scissors representing the internal cleavage site.

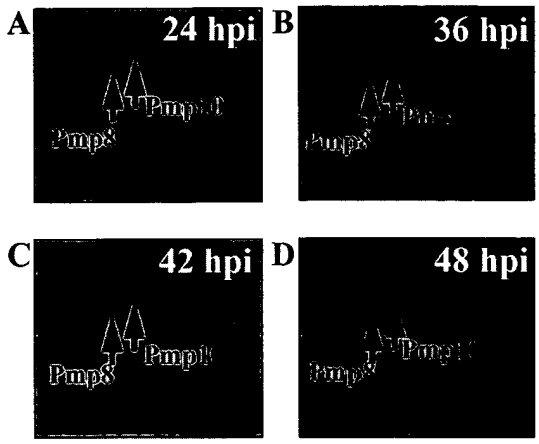

Figure 3
Visualization of Pmp8 and Pmp10 by two-hour labeling and 2-D PAGE Sections from autoradiographs of 2-D PAGE separated proteins from *C. pneumoniae* infected cells labeled with [^{35}S]methionine by two-hour cultivation in a [^{35}S]methionine medium added cycloheximide.

Cleavage at this site results in a fragment of a theoretical Mw of 51 kDa, which is in good agreement with the observed Mw of 48 kDa. For the protein spot at 66 kDa containing the N-terminal peptides, cleavage at the signal peptidase cleavage site predicted by Signal-P was confirmed at the amino acid 30 (^{30}AHSLHSSELD). The theoretical molecular weight of a fragment starting at this position and ending at the cleavage site at amino acid 1185 is 116 kDa. As no peptides were identified at the C-terminal side of amino acid 548 in the N-terminal fragment, further cleavage or degradation from the C-terminal end will be a likely explanation of the discrepancy between expected and observed values.

Pmp20 was identified just above the 90 kDa Pmps, but as no molecular weight markers were present above 90 kDa, the exact molecular weight of the protein cannot be deduced. However, it is found much lower than the expected 178 kDa and the earlier obtained MS identification of this protein spot was based on peptides located in the C-terminal part of Pmp20 (residues 1057–1069, 1387–1400, 1421–1432, 1506–1527, 1557–1571, 1587–1597, and 1644–1654) indicating that also Pmp20 is cleaved.

2-D PAGE analysis of time dependent expression

The time of expression of the Pmps identified in the 2-D protein profile was investigated by pulse labeling with [^{35}S]methionine for two-hour-periods followed by 2-D PAGE and autoradiography. Samples were labeled at 12, 24, 36, 42 and 48 hours post infection (hpi). The earliest time for detection of Pmps was 24 hpi, at which time the highest abundant Pmps in the gels (Pmp8 and Pmp10) were only barely visible (figure 3A). The amount of Pmps expressed in a two-hour labeling period increased during the developmental cycle as illustrated for Pmp8 and Pmp10 in figure 3A,3B,3C,3D. In figure 4 the volumes of Pmp spots (Pmp2, 7, 8, 10, 11, 13, 14 and the C-terminal part of Pmp6) are plotted as percentages of the total spot volumes of the gels at 24, 36 and 48 hpi. The remaining identified Pmps were omitted from this analysis since they could not be confidently quantified due to overlapping spots. Spot volumes were calculated as density integrated over Gaussian area of spots visualized by autoradiography and detected by use of the Melanie II software. The volume of each spot was divided by the number of methionines of the protein. The corrected volume percentages are arbitrary, as the total spot volumes of the gels could not be corrected for methionine content of every protein present in the gel. However, the numbers do reflect the mutual proportion of the measured proteins and the time dependent change in their contribution to the total protein synthesis. Over time, the expression of all the Pmps in figure 4 increased from below 0.05 units at 24 hpi to between 0.15 and 1.35 units at 48 hpi. The most pronounced increase was from 36 to 48 hpi. Pmp10 was found to be expressed at the highest rate (1.5 units at 48 hpi) followed by Pmp8 (0.6 units at 48 hpi). It must be noted that the apparent level of expression is influenced by the properties of the proteins with respect to separation by 2-D PAGE. However, the Pmps must be expected to exhibit comparable behaviour.

A parallel analysis of the expression of MOMP and DnaK was performed for comparison to the Pmps (figure 4). MOMP is expressed during the growth fase of *Chlamydiae* [25]. DnaK is an early protein [26] and has been suggested to be RB specific and hence to show decreased expression late in the developmental cycle [27]. The expression of MOMP was found to increase at the same rate from 24 to 36 hpi as from 36 to 48 hpi (figure 4). The expression of DnaK was similar to that of MOMP at 24 hpi, but showed no increase from 24 to 36 hpi and decreased from 36 to 48 hpi (figure 4).

It can be concluded that all Pmps investigated with respect to time dependent regulation (Pmp2, 6, 7, 8, 10, 11, 13, 14) were found to be strongly up-regulated between 36 and 48 hpi, coinciding with the conversion of RB to EB. This is in agreement with what has been reported for *C. trachomatis* and *C. psittaci* POMPS [28,29]. At 48 hpi Pmp8 and Pmp10 were several fold higher expressed than any other Pmp.

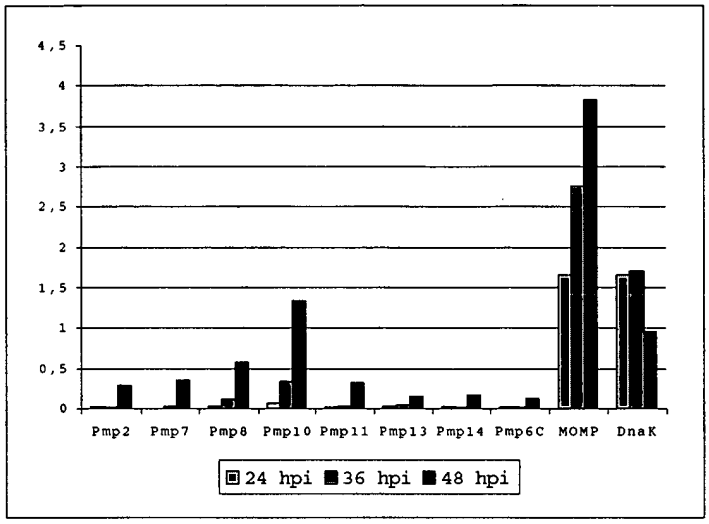

Figure 4
Time dependent expression of Pmps Histogram showing the contribution of selected Pmps to the total amount of protein synthesized in a two-hour labeling period. *Chlamydia* proteins were selectively labeled with [^{35}S]methionine under the addition of cycloheximide to stop host cell protein synthesis, separated by 2-D PAGE and visualized by autoradiography. Infected cells were labeled at 24, 36 and 48 hours post infection. Spots were detected by using the Melaniell software. Spot volumes were calculated as density integrated over Gaussian area, and the percentage of total spot volume in each gel was calculated. The percentages were corrected for the methionine content of each protein. The corrected spot volume percentages are given in arbitrary units at the Y-axis. Only Pmps located in the gel at positions where no overlapping protein spots hindered quantification are depicted. MOMP and DnaK are included as controls.

Immunofluorescence microscopy
In order to investigate whether the Pmps absent from EBs could be detected during growth in cell culture, the antibodies against these Pmps were used in IMF on *C. pneumoniae* infected HEp-2 cells. Cells cultivated on coverslips were infected and fixed in methanol at 72 hpi. The investigated antibodies were visualized with a FITCH conjugated secondary antibody. The antibodies against rPmp15, 16, 18 and 19, which did not react in IMB, did not react in IMF either (table 1). Furthermore, Pab236-rPmp20 was found not to react in IMF (table 1) although Pmp20 was identified in EB gels by IMB and MS. Examples are shown in figure 5.

All Pmp antibodies were used in IMF of infected cells that were formalin fixed at 72 hpi. Formalin cross-linking of outer membrane proteins prevents antibodies from penetrating the bacteria so that only proteins in the outer membrane are accessible. MAb18.1-DnaK was included as a control of the impermeability as described earlier [30]. As the DnaK epitope recognized by MAb18.1 is formalin insensitive [30], the lack of reaction of MAb18.1 (figure 5, row 1) must be due to the prevention of reaction of interior proteins by the fixation.

The pAbs raised against recombinant Pmp1, 2, 7, 13, 14, 15, 16, 19 and 20 did not react after formalin fixation. Due to the described cross-reaction of the Pmp antibodies, the reacting antibodies were absorbed with recombinant proteins. The Pmp antibodies were absorbed with rPmps corresponding to the Pmps to which they cross-reacted in IMB and with the rPmps to which they were raised, respectively. The reaction of pAb208-rPmp9 could be prevented by addition of any of the Pmps 6, 8, 10, 11 or 21 (table 2), and hence it must be considered non-specific. The pAbs raised against rPmp6, 8, 11 and 21 were found to react specifically in formalin fixation (table 2), as their reaction was prevented by the recombinant Pmp that they were raised agains, but not by the ones to which they cross reacted in IMB. Pmp8 is shown as an example in figure 5 (6). Pab203-rPmp10 was not investigated by competition IMF as it was shown to be mono-specific for Pmp10 by IMB.

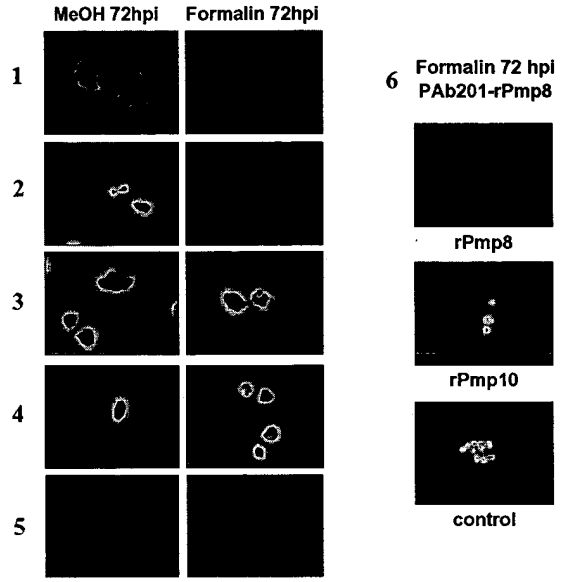

Figure 5
Immunofluorescence microscopy of *C. pneumoniae* infected HEp-2 cells Immunofluorescence microscopy pictures of infected HEp-2 cells fixed in methanol or 3.7% formaldehyde at 72 hours post infection (hpi). Bound antibody was detected with a FITCH conjugated secondary antibody. Fixed cells reacted with **1:** Monoclonal antibody recognizing DnaK (MAb18.1), **2:** pAb116-rMOMP, **3:** pAb110-rOmp2, **4:** pAb237-rPmp21, **5:** pAb234-rPmp19. **6:** Competition IMF. Reaction with pAb201-rPmp8 in the presence of: **rPmp8**: rPmp8, to which the antibody was raised, **rPmp10**: rPmp10, to which the antibody cross reacted in IMB, **control**: no recombinant proteins. As rPmp8 prevents reaction of pAb201-rPmp8 whereas rPmp10 does not affect the reaction, we conclude that the observed reaction is specific for Pmp8.

Pmp10 [30,17] and Pmp11[30] have earlier been shown to be surface exposed and the results indicate that this is also the case for Pmp6, 8 and 21. Other Pmps may also be at the surface, but in that case the epitopes recognized by our antibodies have been destroyed by the fixation or in some way masked.

PAb116-rMOMP did not react after formalin fixation (figure 5, row 2), which is in agreement with earlier reports on the lack of surface exposed linear epitopes of *C. pneumoniae* MOMP [31]. Pab110-rOmp2 did react (figure 5, row 3) indicating the presence of surface exposed epitopes of Omp2 in *C. pneumoniae* in opposition to *C. trachomatis* where Omp2 is probably located at the inner surface of the outer membrane complex [32,33].

Discussion

In the present study, cleavage sites of Pmp6 and Pmp21 have been identified, and it is suggested that also Pmp20 is cleaved. Cleavage of the these Pmps explain why they are found at different molecular weight in different studies. Grimwood *et al.* [19] detected the N-terminal fragment of Pmp6 at 90 kDa that was also identified by Vandahl *et al.* [20], whereas Montigiani *et al.* [21] found the lower molecular weight C-terminal fragment of Pmp6. Only a C-terminal fragment of Pmp21 has been described previously [20,21].

There is no similarity in amino acid sequence between the cleavage sites in Pmp6 and Pmp21, but both sites are located between the C-terminal predicted β-barrel [10] and the N-terminal predicted parallel β-helix fold [34]. This position is in agreement with the theory that the Pmps may be autotransporters; as such often cleave off their N-terminal part [10]. However, if the N-terminal part is liberated it would not be detected in IMB using proteins from purified EBs as antigens, and as the N-terminal fragment is detected in EB gels for Pmp6 and Pmp21 it must be concluded that it remains bound to the EB.

Pab236-rPmp20 was raised against a fragment of Pmp20 covering the N-terminal part and some of the C-terminal fragment, but only the C-terminal part could be detected in IMB (data not shown), suggesting that the N-terminal part may be liberated. Pab236-rPmp20 did not react in IMF. It was raised against unfolded recombinant protein and may not recognize the correctly folded protein, especially not if the N-terminal part is liberated or degraded.

The fragments of Pmp6, Pmp20 and Pmp21 were detected after a labeling period of two hours, and no increase in the amount of cleavage products was observed after a chase period of six hours (data not shown). This suggests that the cleavage occurs rapidly after synthesis. We consider it unlikely that the cleavage should be an artificial phenomenon as the infected cells were harvested in a lysis solution containing 7 M urea, 2 M thiourea and reducing agents, and as the results were highly reproducible. The lack of detection of full-length products of the high molecular weight Pmps may be caused by low resolution in 2-D PAGE of such proteins, but full-length products of these were also absent in 1-D IMB by Grimwood *et al.* [19]. Interestingly, Grimwood *et al.* [24] detected Pmp6 from strains TW183 and AR39 at a Mw of 130 kDa, which is the theoretical Mw of Pmp6 in these strains, due to a deletion of the bases encoding amino acids 429 to 559, which does not include the cleavage site.

In the present study we did not detect any of the Pmps15-18, originating from cluster III. Pmp16 and Pmp18 are the only Pmps lacking a leader sequence. Pmp17 is truncated

Table 2: Competition immunofluorescence

	none	rPmp6	rPmp11	rPmp8	rPmp9	rPmp21	rPmp7	rPmp10
pAb221-rPmp6	+++	0	+++					
pAb195-rPmp11	+++	+	0					
pAb201-rPmp8	+++			0				+++
pAb208-rPmp9	++	0	0	0	0		0	
pAb237-rPmp21	+++	+++				0		

The results of competition IMF in which the recombinant Pmp listed in the top row was added to an IMF reaction using the polyclonal antibodies listed in the first column. The reactions were carried out only for the shaded table cells: **0**, no reaction; **+**, weak reaction; **++**, moderate reaction; **+++**, strong reaction.

and was thus not investigated. Montigiani *et al.* [21] have reported identification of Pmp16 in *C. pneumoniae* from a clinical isolate, but Grimwood *et al.* [19] found that both Pmp16 and Pmp18 were unstable in CWL029, which may explain that these Pmps were not observed in our study. However, it could also be speculated whether differences have been introduced through laboratory passages. We observe expression of Pmp10 in strain CWL029 whereas it was not detected in strain CWL029 by Grimwood *et al.* [19] due to a frame shift mutation. Grimwood *et al.* [19] have described differences in the expression of Pmp1 and Pmp3 between strains CWL029 and TW183 as determined by immunoblotting, meaning that at least some Pmps are differentially expressed between strains.

Pmp6, Pmp8, Pmp10, Pmp11 and Pmp21 were detected at the surface of the bacteria by formalin fixation IMF. Apart from Pmp21, all the Pmps detected at the surface show greatest similarity to PmpG [4], a constituent of the outer membrane of *C. trachomatis* L2 [35,28]. An explanation of the expansion of the number of *C. pneumoniae* Pmps similar to PmpG could be that *C. pneumoniae* varies its surface to escape the immune defence of the host by changing the expression of Pmps. However, if Pmp expression is changed in order to vary the surface, this is most likely a consequence of the surface localization of Pmps rather than the very reason for the existence of Pmps.

The most highly expressed among all the investigated Pmps at all points in time is Pmp10, which is known to contain surface exposed epitopes [30]. Pmp10 is differentially expressed in infected cell culture [17] and mice [16]. Pmp10 still being the most highly expressed Pmp suggests that it carries out an important function. This function would presumably have to be supplemented in bacteria not expressing Pmp10. Pmp10 contains a predicted signal peptidase II cleavage site directing lipid modification and *pmp11*, which is located next to *pmp10* (but in the opposite direction), encodes the only other predicted lipid modified Pmp. If the function of Pmp10 depends on lipid modification, and this function is needed in bacteria lacking Pmp10, Pmp11 would be the obvious alternative.

The finding that all Pmps are heavily upregulated at the time of conversion of RBs into EBs indicates that the function of these is structural but they may also be needed with respect to attachment or entry of EBs. An N-terminal triangular beta-layer motif could provide the bacteria with a shielding lattice and ensure proper spacing to a host cell or an epitope exposed to the complement system. If the lipid modifications of Pmp10 and Pmp11 are used as anchors inserted into the host cell membrane, subsequent action of other entry molecules would probably depend on proper spacing. However, all theories on functions of the Pmps remain very speculative.

Conclusions

The Pmps investigated with respect to time dependent regulation (Pmp2, Pmp6, Pmp7, Pmp8, Pmp10, Pmp11, Pmp13, and Pmp14) were found to be up-regulated late in the developmental cycle. Due to their reaction in formalin fixation we propose that Pmp6, Pmp8, Pmp10, Pmp11, and Pmp21 are surface exposed. Pmp6, Pmp20 and Pmp21 were found in cleaved forms and the identified cleavage sites of Pmp6 and Pmp21 are in agreement with the theory that the Pmps are autotransporters.

Methods

Organisms and cultivation

C. pneumoniae CWL029 (also known as VR1310) (ATTC) was cultivated in semi confluent monolayers of Hep-2 cells (ATCC) as described [20]. Elementary bodies were purified 72 hpi essentially as described [30] with the exception that Visipaque replaced Urografin for gradient making.

PCR and cloning

PCR enzymes were Expand™ High Fidelity (Roche) and reaction conditions were as recommended by the manufac-

turer. As the Ligation-Independent Cloning (LIC) kit (Novagen) was used for cloning of PCR products, all primers (DNA Technology) were designed with LIC specific 5'-sequences: Forward primer: 5' GAC GAC GAC AAG AT *pmp*-sequence 3'. Reverse primer: 5' GAG GAG AAG CCC GGT *pmp*-sequence 3'. Primers for amplification of *pmp* genes were placed as listed in table 1 and the sequences were obtained from GeneBank (AE001363). In those cases where only the N-terminal of the Pmp gene was cloned a stop codon was introduced. The pET 30Ek LIC vector (Novagen) was used for cloning and expression of recombinant protein according to the protocol provided by the manufacturer. Cloning was performed in *E. coli* NovaBlue.

Protein expression and purification

Plasmid DNA was prepared from NovaBlue cells by the alkaline lysis method and used for transformation of *E. coli* BL21(DE3) by eletroporation. Clones were tested for correct insert sequence by PCR with vector specific primers. The PCR product was used for sequence reactions using the Terminator Ready Reaction Mix (Perkin Elmer), and sequencing performed on an ABI PRISMTM 377 DNA Sequencer (Perkin Elmer). IPTG (1 mM) induction of expression of His-tagged fusion proteins was performed for two hours at 37°C when A_{600} reached 0.4. Recombinant protein was purified from pelleted cells using HiTrap Ni^{2+} columns (Amersham Pharmacia). The purity of the recombinant proteins was tested by SDS PAGE and the protein concentration measured by use of the Bradford Protein Assay (BioRad).

Production of antibodies

New Zealand White rabbits were immunized: i) intramuscularly on days 1, 8 and 15 using 10 μg of protein in PBS and 50% Freunds incomplete adjuvant; ii) intravenously on days 29, 36 and 43 using 10 μg of protein in PBS. The rabbits were bled on day 60.

Labeling

Pulse labeling of infected cell cultures was performed by using 100 μCi/mL radioactive methionine (Amersham Pharmacia) in a methionine-free RPMI 1640 medium as described previously [20]. 40 μg/mL cycloheximide was added to inhibit host cell protein synthesis during labeling. Cell cultures were labeled for two-hour-periods at 6, 12, 24, 26, 42, 48 and 54 hpi and harvested immediately after labeling by scraping off in lysis solution (7 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM Tris base, 65 mM DTE and 2% v/v Pharmalyte 3–10 (Amersham Pharmacia)). Before purification of EB the same labeling periods were used to ensure incorporation of radioactivity into proteins synthesized at all times during the developmental cycle.

Electrophoresis

One-dimensional SDS gels were run as described [30]. The protocol for two-dimensional gel electrophoresis was as described [20]. First dimension was carried out using non-linear Immobiline Drystrips pH 3–10 (Amersham Pharmacia). Proteins were focused in the strips using an IPGphor (Amersham Pharmacia) applying a voltage of 20 V for rehydration of strips and 120 kVh for focusing. For second dimension 9–16% T gradient polyacrylamide SDS gels were used. Comparative gels were loaded with 300.000 cpm as determined by TCA precipitation and scintillation counting. Gels for N-terminal sequencing were loaded with 600 μg protein from purified unlabeled EB. Gels for MS identification were loaded with 3.000.000 cpm and a total of 600 μg protein – all from purified EB. Gels for IMB were loaded with 75 μg protein from purified unlabeled EB and 300.000 cpm labeled EB protein. Protein spots in gels were visualized by autoradiography as described [20]. Comparative gels were treated with Amplify (Amersham Pharmacia) before exposure to BioMax MR X-ray films (Kodak).

Immunoblotting

Two-dimensional gels were washed for five min. in double-distilled water and soaked for 30 min. in transfer buffer. PVDF membranes (Immobilon-P, pore size 0.45 μm, Millipore) were soaked for one min. in methanol and then for 30 min. in transfer buffer. The transfer buffer contained: 50 mM Tris, 50 mM boric acid, 0.02% SDS and 10% methanol. The transfer was performed at 90 V and 10°C for four hours. Membranes were blocked in a buffer containing 20 mM Tris, 150 mM NaCl, and 3% gelatine, pH 7.5, washed in washing buffer (20 mM Tris, 500 mM NaCl, 0.05% tween-20) and incubated with polyclonal antibodies (pAbs) diluted 1/300 in antibody buffer (washing buffer added 0.2% gelatine) for one hour at 37°. After three washing steps membranes were incubated with secondary antibody (goat-anti-rabbit-IgG-AP-conjugate, BioRad) diluted 1/2000 for another hour at 37°C. After washing the blots, bound antibody was visualized with 270 μL NBT (50 mg/mL in 70% DMF) and 270 μL BCIP (25 mg/mL in 100% DMF) in 45 mL buffer containing 100 mM NaCl, 5 mM MgCl and 100 mM Tris-HCl at pH 9.5. As a fraction of the EB proteins was radiolabeled, reacting spots could be identified by comparison to a reference gel [20] upon autoradiography of the PVDF membrane [22].

Mass spectrometry

Protein spots excised from preparative gels were identified by MALDI TOF mass spectrometry as described [20].

N-terminal sequencing

Protein spots excised and pooled from eight Coomassie Brilliant Blue stained PVDF membranes each loaded with

600 μg of protein from purified EB were analyzed using an Applied Biosystems 404 protein sequencer (Perkin Elmer).

Immunofluorescence microscopy

IMF was performed as described [36] analyzing infected cells fixed in methanol or formalin at 72 hpi. Formalin fixed cells were permeabilized with 0.2% Triton X-100 for 10 minutes at room temperature. Bound primary antibody was visualized with a secondary FITCH conjugated antibody (DAKO), which was absorbed to prevent cross-reaction.

Authors' contributions

B.B.V. carried out the 2-D PAGE, IMB and IMF experiments, analyzed the Edman degradation results and drafted the manuscript. A.S.P. made the recombinant Pmps. K.G. and J.V. carried out the MS analysis. A.H. carried out the Edman degradation. G.C and S.B. participated in design and coordination of the study. All authors read and approved the final manuscript

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